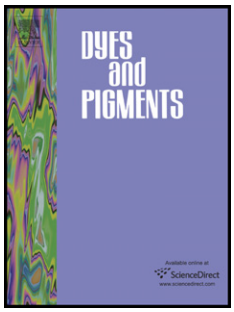

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Research Highlights

1. The phthalocyanines water solubility is related to the proportion of free hydroxyl groups.

2. The cell interaction changes according to the hydrophobic to hydrophilic balance.

3. Unprotected derivatives presented a greater tendency to aggregate but higher cell interaction.

Synthesis of unsymmetrical phthalocyanine derivatives and their interaction with mammary MCF7 cells

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Abstract

We report herein the synthesis and characterization of a group of unsymmetrical phthalocyanines modified with different combinations of hydrophobic and hydrophilic groups. Our results demonstrated that their solubility in polar media varies with the proportion of hydroxylated groups present in each structure and that the cell interactions and generation of reactive oxygen species in aqueous media change according to the hydrophobic to hydrophilic balance in the macrocycle.

Keywords: phthalocyanines, synthesis, photodynamic therapy, cell interaction, glycerol

1. Introduction

Photodynamic therapy (PDT) is a technique that combines a photosensitizer, oxygen and light to treat diseases such as cancer, acne, and psoriasis [1].This treatment uses a photosensitizer that is nontoxic in the dark to focus the therapeutic action to only those regions irradiated by light [2, 3].

Of the approved and studied photosensitizers for this treatment [4], phthalocyanine derivatives are the most promising due to their high molar extinction coefficient in the 600–800 nm region and their high singlet oxygen quantum yields under irradiation [5, 6]. However, the primary limitation for their use is their low solubility in water and physiological fluids due to their tendency to aggregate in aqueous media [7].

Recently, various strategies to obtain phthalocyanine derivatives with better solubility in aqueous media have been reported. These methods primarily involve the incorporation of cationic [8] and anionic [9, 10] groups, peptides [11], β-cyclodextrins [12, 13] or crown ethers [14] in the phthalocyanine ring to diminish the aggregation of the compound via either spatial conformation or columbic forces.

Herein, we present a photochemical and photophysical study in polar media of a group of unsymmetrical phthalocyanines bearing polyhydroxyl moieties, as well as their interaction with mammary MCF7 cells. The properties of unsymmetrical phthalocyanine structures have been explored in the literature [15], with some examples of carbohydrate substituted phthalocyanines studied for PDT applications [16]. But only a few works present the synthesis, characterization and biological studies of phthalocyanines with different proportions of polyhydroxylated groups in their macrocycle structure [17]. Also, some studies involving porphyrin and chlorin derivatives have reported that the combination of hydrophobic and hydrophilic groups within the same molecule results in improved intramolecular polarity, which can facilitate cell membrane penetration [18]. The primary goal with the phthalocyanine derivatives studied here is to compare the cell interactions of related macrocycles substituted with different proportions of protected and unprotected hydroxyl groups (-OH) which promotes change in polarity.

2. Experimental Section.

2.1 General considerations

The 1,2-dicyanobenzene (ii) and 4-methyl-1,2-dicyanobenzene (iii) were purchase from Aldrich and used without purification. Solketal was synthesized as described in literature [19] and the structure confirmed by ^{1}H NMR. When necessary, solvents were purified following standard literature procedures. Some reactions were carried out under nitrogen atmosphere as specified in the experimental procedures. In ^{1}H NMR spectra (500 MHz), $CDCl_3$, DMSO-d6 solventes and tetramethylsilane were used. The chemical shifts are expressed

in δ (ppm). Flash chromatography was carried out by using silica gel (230-400 mesh). Analytical TLC was carried out on precoated sheets with silica gel (0.2 mm thick). Mass spectra were recorded by HMRS-MALDI-TOF (Bruker-Daltonics, USA).

2.2. *Synthesis of 4-solketalphthalonitrile* **(i)**

Solketal (102 g, 7.75 mmol) and 4-nitrophthalonitrile (1.34 g, 7.75 mmol) were stirred in a three-necked flask for three days in DMF (6 mL) at room temperature in the presence of an excess of potassium carbonate (7 g). The mixture was poured in ice water, extracted with dichloromethane (DCM) (3 x 30 mL) and washed with distilled water (3 x 50 mL). The organic phase was dried over anhydrous magnesium sulfate anhydrous, filtered and concentrated under reduced pressure. The product was purified by column chromatography (silica gel: hexane-ethyl acetate 1:1 v / v) resulting in a yellow solid which was finally purified by recrystallization with methanol, yielding a white solid, 67% yield. FTIR (KBr) u_{max}/cm^{-1} 3072 (Ar–CH, w), 2994, 2062, 2890 (–CH, $–CH_2$, and $–CH_3$, w), 2231 (–C≡N, w), 1600, 1498 (-C=C-, s), 1255, 1033 (C-O-C). ^{1}H NMR ($CDCl_3$): δ (ppm) 7.73 (d, *J*=8.8 Hz, 1H), 7.32 (d, *J*=2.7 Hz, 1H), 7.25 – 7.22 (dd, *J*=8.8 Hz and *J*=2.7 Hz, 1H), 4.50 (m, 1H), 4.19 (m, 1H), 4.09 (m, 2H), 3.89 (m, 1H), 1.45 (s, 3H, CH_3), 1.37 (s, 3H, CH_3). HRMS-MALDI-TOF m/z calcd for $C_{14}H_{14}N_2O_3$ $(M+Na)^+$ 281.0902, found 281.0896.

*2.3. Synthesis of symmetrical phthalocyanines **1** and **2***

2.3.1. *Synthesis of [tetra(solketal)phthalocyaninate]zinc(II)* **(1)**

In a closed reaction tube, 4-solketalphthalonitrile (i, 800 mg, 3.09 mmol) and zinc acetate (339 mg, 1.54 mmol) were heated under reflux for 10 h in 5 mL of *N,N*-Dimethylethanolamine , under nitrogen atmosphere, with constant stirring. After cooling, the reaction mixture was concentrated under reduced pressure and the dark blue crude product was purified by column chromatography (silica gel: gradient elution, hexane–ethyl acetate (2:1) to pure ethyl acetate), giving the compound (**1**) in 72% yield (667 mg; 0.557 mmol). mp > 300 °C. FTIR (KBr) u_{max}/cm^{-1} 3066 (Ar–CH, w), 2983, 2933, 2873 (–CH, $–CH_2$, and $–CH_3$, w), 1733 (–N=C–, s), 1606, 1488 (-C=C-, s), 1236, 1040 (C-O-C). UV-Vis (DMSO), λmax, (log ε): 358 (4.72), 613 (4.36), 682 (5.05). ^{1}H NMR ($CDCl_3$): δ 7.73 (d, *J*=8.4 Hz, 4H), 7.31 (d, *J*=2.2 Hz, 4H), 7.21 (dd, *J*=8.4 Hz and *J*=2.2 Hz

4H), 4.50 – 4.48 (m, 4H), 4.19 – 4.11 (m, 8H), 4.07 – 4.04 (m, 4H), 3.92 – 3.89 (m, 4H), 1.46 (s, 12H, -CH_3), 1.41 (s, 12H -CH_3). HRMS-MALDI-TOF m/z calcd for $C_{56}H_{56}N_8O_{12}Zn^+$ $(M)^+$ 1096.3310, found 1096.3321.

2.3.2. Synthesis of [tetra(glyceryl)phthalocyaninate]zinc(II) **(2)**

Compound **1** (200 mg, 0.418 mmol) was stirred overnight in excess of acetic acid 80% solution. The reaction mixture was then evaporated to dryness under reduced pressure and the dark blue powder was washed successively with ethyl acetate, hexane, dichloromethane and chloroform to yield the compound **2** as a dark blue powder with 94.,4 % yield (147.7 mg ; 0.157 mmol). mp > 300 °C. FTIR (KBr) υ_{max}/cm^{-1} 3367 (–OH, s) 3068 (Ar–CH, w), 2937, 2881 (–CH and –CH_2, w), 1733 (–N=C–, s), 1616, 1490 (-C=C-, s), 1228, 1041 (C-O-C). UV-Vis (DMSO), λmax, (log ε): 358 (4.89), 614 (4.54), 682 (5.23). ^{1}H NMR (DMSO-d_6): δ 9.13 – 9.04 (m, 3H), 8.73 – 8.62 (m, 3H), 7.73 – 7.66 (m, 4H), 7.35 – 7.28 (m, 2H), 5.33 – 5.18 (m, 3H), 5.10 – 4.88 (m, 5H), 4.65 – 4.58 (m, 3H), 4.56 – 4.45 (m, 3H), 4.21 – 3.98 (m, 6H), 3.85 – 3.68 (m, 8H, -OH). HRMS-MALDI-TOF m/z calcd for $C_{44}H_{40}N_8O_{12}Zn^+$ $(M)^+$ 936.2057, found 936.2052.

2.4. Synthesis of Non symmetrical (A_3B) phthalocyanines ***(3)***, ***(4)***, ***(5)*** *and* ***(6)***

2.4.1. Synthesis of [tris-2,9(10),16(17)-solketal-phthalocyaninate]zinc(II) ***(3):*** **i** (1.81 g, 702 mmol) and ii (300 mg, 2.34 mmol) was heated under reflux in *N,N*-dimethylethanolamine (6 mL), under a nitrogen atmosphere, for 12h, in the presence of excess of zinc acetate. After reaction, the solution was cooled and the product was precipitated and washed with water/methanol (10:1), and dried under vacuum. The compound was purified by column chromatography (silica gel, toluene/THF gradient 95/5 to 50/50) as a green solid in 8% yield (181 mg; 0.187 mmol). mp > 300 °C. FTIR (KBr)υ_{max}/cm^{-1} 3062 (Ar–CH, w), 2979, 2923, 2852 (–CH, –CH_2 and –CH_3, w), 1608, 1488 (-C=C-, s), 1340 (-C=N-), 1226, 1040 (C-O-C). UV-Vis (DMSO), λmax, (log ε): 356 (4.71), 612 (4.38), 680 (5.09). ^{1}H NMR ($CDCl_3$): δ 7.78 – 7.70 (m, 4H), 7.27 - 7.22 (m, 9H), 4.57 – 4.50 (m, 3H), 4.23 – 4.13 (m, 6H), 4.11 – 4.05 (m, 3H), 3.96 – 3.89 (m, 3H), 1.48 (s, 9H, -CH_3), 1.42 (s, 9 H, -CH_3). HRMS-MALDI-TOF m/z calcd for $C_{50}H_{46}N_8O_9Zn^+$ $(M)^+$ 966.2679, found 966.2679.

Synthesis of [tris-2,9(10),16(17)-glyceryl-phthalocyaninate]zinc(II) ***(4)****:* Compound (3) (80 mg, 0.0827 mmol) was stirred overnight in excess of acetic acid 80% solution. The purification procedure was treated under the same conditions used for compound (2). Yield 87.5% (181 mg; 0.187 mmol). mp > 300 °C. FTIR (KBr)υ_{max}/cm^{-1} 3421 (–OH, s), 3063 (Ar–CH, w), 2981, 2931 (–CH and –CH_2, w), 1731 (–N=C–, s), 1606, 1486 (-C=C-, s), 1333, 1289 (-C=N-), 1232, 1054 (C-O-C). UV-Vis (DMSO), λmax, (log ε): 357 (4.79), 612 (4.46), 680 (5.17). ^{1}H NMR (DMSO-d_6): δ7.73- 7.65 (m, 4H), 7.35 - 7.26 (m, 9 H), 5.10 – 4.98 (m, 3H), 4.76 – 4.66 (m, 3H), 4.20 – 4.02 (m, 6H), 3.84 – 3.80 (m, 3H), 3.54 – 3,38 (m, 6H, -OH). HRMS-MALDI-TOF m/z calcd for $C_{41}H_{34}N_8O_9Zn^+$ $(M)^+$ 846.1740, found 846.1743.

Synthesis of [tris-2,9(10),16(17) solketal-23(24)-mono(methyl)phthalocyaninate]zinc(II) ***(5):*** i (1.63 g, 6.33 mmol) and iii (300 mg, 2.11 mmol) was treated under the same conditions adopted for compound (3). Yield 13% (269 mg ; 0.270 mmol). mp > 300 °C. FTIR (KBr)υ_{max}/cm^{-1} 3062 (Ar–CH, w), 2923, 2857 (–CH, –CH_2, and –CH_3, w), 1608, 1465 (-C=C-, s), 1378, 1330 (-C=N-, -C–N=), 1240 (C-O-C). UV-Vis (DMSO), λmax, (log ε): 356 (4.50), 613 (4.16), 681 (4.87). ^{1}H NMR ($CDCl_3$): δ7.78 - 7.69 (m, 4H), 7.56 - 7.47 (m, 1H), 7.33 - 7.22 (m, 4H), 7.21 - 7.13 (m, 3H), 4.53 - 4.45 (m, 3H), 4.20 - 4.15 (m, 3H), 4.13 - 4.08 (m, 3H), 4.07 - 4.02 (m, 3H), 3.96 - 3.82 (m, 3H), 2.52 - 2.42 (m, 3H, Ar-CH_3), 1.46 (s, 9H, -CH_3), 1.40 (s, 9H, -CH_3). HRMS-MALDI-TOF m/z calcd for $C_{51}H_{48}N_8O_9Zn^+$ $(M)^+$ 980.2836, found 980.2834.

Synthesis of [tris-2,9(10),16(17)- glyceryl-23(24)-mono(methyl)phthalocyaninate]zinc(II) ***(6):*** Compound (5) (130 mg, 0.132 mmol) was stirred overnight in excess of acetic acid 80% solution. The purification procedure was the same used for compound (2). Yield 80.7% (141 mg ; 0.164 mmol). mp > 300 °C. FTIR (KBr)υ_{max}/cm^{-1} 3421 (–OH, s), 3004 (Ar–CH, w), 2917 (–CH, –CH_2 and –CH_3, w), 1648, 1485 (-C=C-,w), 1333 (-C=N-), 1236, 1022 (C-O-C). UV-Vis (DMSO), λmax, (log ε): 356 (4.66), 613 (4.32), 681 (5.03). ^{1}H NMR (DMSO-d_6): δ7.73 – 7.66 (m, 4H), 7.34 - 7.26 (m, 8H), 5.11 - 5.01 (m, 3H), 4.83 - 4.64 (m, 3H), 4.22 - 4.15 (m, 3H), 4.08 - 4.01 (m, 3H), 3.85 - 3.79 (m, 3H), 3.50 - 3.42 (m, 6H, -OH), 2.51 (s, 3H, Ar-CH_3). HRMS-MALDI-TOF m/z calcd for $C_{42}H_{36}N_8O_9Zn^+$ $(M)^+$ 860.1897, found 860.1905.

2.5. Unsymmetrical (AB_3) phthalocyanines ***(7)****,* ***(8)****,* ***(9)*** *and* ***(10)***

2.5.1. Synthesis of [mono(solketal)phthalocyaninate]zinc(II) ***7:*** i (300 mg, 1.16 mmol) and ii (0.446 g, 3.48 mmol) was heated under reflux in *N,N*-dimethylethanolamine (6 mL), under a nitrogen atmosphere, for 12h, in the presence of excess of zinc acetate. After that, the solution was cooled and the product was precipitated and washed with water/methanol (10:1) and dried under vacuum. The compound was purified by column chromatography (silica gel, toluene/THF) to yield **7** as a green solid in 12% yield (98 mg ; 0.139 mmol). mp > 300 °C. FTIR (KBr)υ_{max}/cm^{-1} 3128, 3068 (Ar–CH, w), 2983, 2933, 2877 (–CH, –CH_2, –CH_3 w), 1722 (–N=C–, s), 1608, 1488 (-C=C-, s), 1336, 1124 (-C=N-), 1228, 1040 (C-O-C). UV-Vis (DMSO), λmax, (log ε): 350 (4.79), 608 (4.55), 674 (5.32). ^{1}H NMR (DMSO-d_6): δ 9.22 - 9.14 (m, 3H), 9.12 - 9.08 (m, 2H), 9.07 - 9.01 (m, 2H), 8.78 -8.64 (m, 1H), 8.02 - 8.22 (m, 6H), 7.48 - 7.45 (m, 1H), 4.78 - 4.71 (m, 1H), 4.58 - 4.50 (m, 2H), 4.47 - 4.41 (m, 1H), 4.22 - 4.18 (m, 1H), 1.62 (s, 3 H, -CH_3), 1.49 (s, 3 H, -CH_3). HRMS-MALDI-TOF m/z calcd for $C_{38}H_{26}N_8O_3Zn^+$ $(M)^+$ 706.1422, found 706.1436.

Synthesis of [mono(glyceryl)phthalocyaninate]zinc(II) ***(8):*** Compound **7** (49 mg, 0.0073 mmol) was stirred overnight in excess of acetic acid 80% solution. The purification procedure was the same used for compound (2). Yield 91.6% (42.3 mg ; 0.0635 mmol). mp > 300 °C. FTIR (KBr)υ_{max}/cm^{-1} 3405 (–OH, s), 3004 (Ar–CH, w), 2919 (–CH and –CH_2, w), 1718 (–N=C–, s), 1608, 1484 (-C=C-, w), 1333, 1231 (-C=N-). UV-Vis (DMSO), λmax, (log ε): 354 (4.76), 612 (4.45), 679 (5.18). ^{1}H NMR (DMSO-d_6): δ 8.23 - 8.11 (m, 5H), 7.86 - 7.78 (m, 1H), 7.76-7.69 (m, 4H), 7.40 - 7.24 (m, 5H), 5.01 - 4.93 (m, 1H), 4.77- 4.68 (m, 2H), 4.65 - 4.57 (m, 1H), 4.55 - 4.46 (m, 1H), 3.49 - 3.43 (m, 2H, -OH). HRMS-MALDI-TOF m/z calcd for $C_{35}H_{22}N_8O_3Zn^+$ $(M)^+$ 666.1106, found: 666.1108.

Synthesis of [tris-2,9(10),16(17)-methyl-23(24)-solketal-phthalocyaninate]zinc(II) **(9):** i (300 mg, 1.16 mmol) and iii (494 mg, 3.48 mmol) was treated under the same conditions adopted for compound **7**. Yield 15% (130 mg ; 0.174 mmol). mp > 300 °C. FTIR (KBr)υ_{max}/cm^{-1} 3128, 3068 (Ar–CH, w), 2983, 2933, 2877 (–CH, –CH_2, –CH_3, w), 1703 (–N=C–, w), 1610, 1488 (-C=C-, s) 1328, 1271 (-C=N-), 1236, 1046 (C-O-C). UV-Vis (DMSO), λmax, (log ε): 353 (4.53), 612 (4.22), 679 (5.00). ^{1}H NMR (DMSO-d_6): δ 7.72 – 7.67 (m, 4 H), 7.65 - 7.59 (m, 8H), 4.80 - 4.73 (m, 1H), 4.60 - 4.52 (m, 2H), 4.44 - 4.36 (m, 1H), 4.19 - 4.11 (m, 1H), 2.51 -

2.48 (m, 9H, Ar-CH_3), 1.62 (s, 3H, -CH_3), 1.50 (s, 3H, -CH_3). HRMS-MALDI-TOF m/z calcd for $C_{41}H_{32}N_8O_3Zn^+$ $(M)^+$ 748.1889, found 748.1901.

Synthesis of [tris-2,9(10),16(17)-methyl-23(24)-glyceryl-phthalocyaninate]zinc(II) ***(10):*** Compound **9** (65 mg, 0.0868 mmol) was stirred overnight in excess of acetic acid 80% solution. The purification procedure was the same used for compound **2**. Yield 94% (57.8 mg ; 0.081 mmol). mp > 300 °C. FTIR (KBr)υ_{max}/cm^{-1} 3444 (–OH, s), 3087 (Ar–CH, w), 2981, 2917, 2865 (–CH, $–CH_2$, and $–CH_3$, w), 1733 (–N=C–, w) 1606, 1488 (-C=C-,w), 1333, 1271(-C=N-), 1226, 1093 (C-O-C). UV-Vis (DMSO), λmax, (log ε): 353 (4.62), 612 (4.31), 680 (5.06). ^{1}H NMR (DMSO-d_6): δ 7.96 - 7.79 (m, 3H), 7.74 - 7.58 (m, 6H), 7.36 - 7.11 (m, 3H), 4.70 - 4.51 (m, 1H), 4.55 - 4.41 (m, 1H), 4.23 - 4.14 (m, 2H), 4.08 - 3.96 (m, 1H), 2.49 (m, 9H, Ar-CH_3). HRMS-MALDI-TOF m/z calcd for $C_{38}H_{28}N_8O_3Zn^+$ $(M)^+$ 708.1370, found 708.1371.

2.6. Photophysichal and photochemical studies

2.6.1. Singlet oxygen quantum yields

All emission, excitation and electronic absorption spectra of the phthalocyanines were recorded in 10 mm path length fluorescence cuvettes in DMSO. Photogeneration quantum yields of singlet oxygen (Φ_Δ) were obtained by indirect method, using diphenylisobenzofuran (DBPF) [20, 21] as chemical quencher. Typically, a mixture of the phthalocyanine (absorption ~0.2 in 680 nm) and the DPBF (absorption ~ 1.2 in 418 nm) was irradiated with a red LED lamp (23 mW) in 20 cycles of 6 seconds each one. The Φ_Δ values were determined using zinc phthalocyanine (ZnPc) (Eq.1):

$$\Phi_\Delta = \Phi_\Delta^{PcZn} \frac{W \; x \; I_{abs}^{PcZn}}{W^{PcZn} \; x \; I_{abs}}$$

Where Φ_Δ^{PcZn} is the singlet oxygen quantum yield for the ZnPc (Φ_Δ= 0.67 in DMSO [20]); **W** and $\mathbf{W^{PcZn}}$ is the DPBF photobleaching rates in the presence of the phthalocyanine and ZnPc, respectively (this value was

obtained by the first exponential curves using the Origin® program); $\mathbf{I_{abs}}$ and $\mathbf{I_{abs}^{PcZn}}$ are the light absorption rates by the phthalocyanines and ZnPc, respectively.

2.6.2. Fluorescence quantum yields

Fluorescence quantum yields ($\mathbf{\Phi_F}$) of all compounds were determined by comparative method using the following equation (Eq.2):

$$\Phi_F = \Phi_F(\text{ZnPc}) \frac{F \cdot A_{ZnPc} \cdot \eta^2}{F_{ZnPc} \cdot A \cdot \eta^2_{ZnPc}}$$

Were F and $\mathbf{F_{ZnPc}}$ are the areas under the fluorescence emission curves of the pthalocyanines (1 – 10) and the standard, respectively. **A** and $\mathbf{A_{ZnPc}}$ are the relative absorbance of the sample and standard at the excitation wavelength, respectively. $\boldsymbol{\eta}^2$ and $\boldsymbol{\eta}^2_{\mathbf{ZnPc}}$ are the refractive indices of the solvents for the sample and standard, respectively. The unsubstituted ZnPc (Φ_F(ZnPc)=0.20) [21] was employed as the standard. The solutions of the compounds were prepared in DMSO with absorbance ranged between 0.28 and 0.35 at the excitation wavelength.

2.7. Determination of partition coefficients ($P_{O/W}$)

Five milliliters of 10 μM solution from each phthalocyanine derivative was prepared in *n*-octanol. The UV-vis spectrum of solution was sampled. Then water (5 mL) was added to solution and the container was stirred for 30 minutes. The centrifugation (5 minutes at 5000 rpm) enabled a phase separation and the organic phase was sampled again. The partition coefficient was obtained from the difference in the phthalocyanine absorption intensity (in 650 nm region) in both stages. At least three independent measures were performed and the corresponding $P_{O/W}$ value was taken as the overall average.

2.8. Cell interaction studies

Cell culture – Cell culture was manipulated using sterile disposable non-pyrogenic plastic ware and were maintained at 37° C in an atmosphere of 5% CO_2 in air at a relative humidity of 80%. Human breast epithelial cells MCF-7 (adenocarcinoma, estrogen receptor +; ATCC**)** were incubated in RPMI-1640 medium (Sigma)

supplemented with 10% fetal bovine serum (Gibco), 10.0 µg/mL insulin purified from bovine pancreas (Sigma), 100 U/mL penicillin and 10.0 µg/mL streptomycin (Sigma). Cells were routinely trypsinized and reinoculated onto plates at a density of 4 x 10^4 cells/cm^2.

2.9. Fluorescence microscopy

Phthalocyanines formulated in DMSO solution were diluted in fresh cell medium to give a 45.0 µM concentration and incubated with MCF-7 cells. MCF-7 cells were grown on chamber slides at a density of 4 x 10^4 cells/cm^2 and then treated with 300 nM DAPI solution (4',6-diamidino-2-phenylindole, Invitrogen) at 37 °C for 30 minutes. The cells were then washed with phosphate buffered saline (PBS: 137 mM NaCl and 2.7 mM KCl in 10 mM phosphate buffer at pH 7.4), and incubated with 45 µM phthalocyanines in dark. After 1-h treatment, the cells were pre-fixed with 30% and 50% ethanol diluted in cells medium (v/v) followed by a 100% ethanol post-fixation. The cells were then washed with phosphate buffered saline (PBS). The fluorescence images were produced by 340 nm *excitation, using a* 580 nm emission filter in a AXIO – observer A1 fluorescence microscope, Zeiss. DAPI was used due their interaction with cell nuclei and its blue fluorescence, allowing following the positions of studied phthalocyanine (red fluorescence) related to the nuclei.

2.10. Flow cytometry Assay

The percentage of phthalocyanines entrance was determined quantitatively by flow cytometry. MCF-7 cells were seeded in 12-well plates and incubated with 1 h with 45.0 µM phtalocyanines. The cells were harvested and washed with ice-cold PBS buffer for 3 times. The cell pellet was resuspended in 200 µL of PBS buffer and the flow cytometric analysis (Cytometer FC 500 MPL – Beckman Coulter) was performed to determine the percentage of phthalocyanines entrance in each sample using a 365 nm arc lamp.

3. Results and discussion

3.1. Preparation and structural characterization

Phthalocyanine **1** (Fig. 1) was synthesized by heating 4-solketalphthalonitrile in the presence of zinc acetate and a solvent (*N*,*N*-dimethylethanolamine) [22]. To remove the acetal group, compound **1** was stirred for 24 h in acetic acid (80% solution) at room temperature, which resulted in the precipitation and isolation of **2** in 68% yield. These two compounds were synthesized to support the comparative study of unsymmetrical phthalocyanines with similar moieties.

The new unsymmetrical phthalocyanines **3–10** were prepared via the statistical cyclotetramerization of a mixture of two different phthalonitriles. We used a 3-fold excess of 4-solketalphhtalonitrile versus 1,2-dicyanobenzene or 4-methylphthalonitrile to obtain A_3B-phthalocyanines **3** and **5**. The AB_3-phthalocyanines **7** and **9** were synthesized using the reverse molar excess combination of the same phthalonitriles. The applied conditions led to a mixture of different macrocycles that were purified via silica-gel column chromatography. Phthalocyanines with free hydroxyl groups were obtained by stirring the macrocycles in an acetic acid solution.

<Fig. 1>

All of the synthesized structures were confirmed by ^{1}H NMR and HRMS. The presence (and integration) of signals in the region between 4.0-5.0 ppm and 1.40-1.62 ppm in all of the ^{1}H NMR spectra confirmed the solketal derivative proportions in the structure (Fig 2). Disappearance of the acetal signals for (2), (4), (6), (8) and (10) in the NMR spectrum in the region between 1.40-1.62 ppm confirmed the efficiency of the deprotection reaction conditions used to convert of the solketal groups into glycerol moieties. It should be noted that each phthalocyanine derivative presented in this study is a mixture of the expected constitutional isomers.

<Fig. 2>

3.2. Photophysical and photochemical properties

The UV/Vis absorption spectra of compounds 1 -10 presented behavior typical of nonaggregated phthalocyanines in DMSO as confirmed by the intense, narrow Q-band in the red visible region between 674

and 682 nm (Fig. 3) (Table 1). Additionally, by comparing the absorbance spectra, it is possible to observe a slight red shift in the maximum absorption in the Q band region for compounds with three or more hydroxylated derivatives. This result is in accordance with theoretical studies [7] that show the linear combination of atomic orbitals (LCAO) is lower when phthalocyanines are modified in the β peripheral position.

<Fig. 3>

<Table 1>

Phthalocyanines have great tendency to aggregate in aqueous media, which decreases their efficient generation of reactive oxygen species [5]. Such aggregation is explained by strong π–π electron interactions between the planar phthalocyanine rings and can be diminished by introducing peripheral substituents with distinct characteristics.

For macrocycles studied here with at least three peripheral glyceryl groups, we observed a predominance of the monomeric species in solution until a 4:6 Water:DMSO mixture, which can be verified by following the relative intensities of the Q-band in the absorption spectra (Fig.4).

<Fig. 4>

However, even phthalocyanine derivatives with three unprotected groups presented a higher tendency to aggregation in increased aqueous conditions, i.e. 6:4 Water:DMSO media (observed by Q-band relatively intensities). This behavior can be associated to the fact that free hydroxyl groups can both promote better macrocycle solvation in water and an enhancement in hydrogen bonding interactions, which increases the

tendency of the ring to aggregate. Kimura *et al.* [23] have already presented results showing the self-organization of some phthalocyanines derivatives containing one glyceryl group in the structure due to the intermolecular diol hydrogen bonding.

To complement, our UV-Vis absorbance related studies have shown that, at same conditions, the macrocycles with only one glyceryl unprotected moiety presented higher aggregation tendency than macrocycles with three unprotect moieties around the ring. This fact demonstrates the influence of the balance between the water solvation and the hydrogen bonding interactions on the equilibrium between monomers, dimers and oligomers in solution.

3.3. In vitro photodynamic activities

The interaction of phthalocyanines with cells was evaluated using fluorescence microscopy and flow cytometry. In the fluorescence microscopy experiments, the nuclei of MCF-7 tumor cells were labeled with DAPI, which fluoresces blue, to evaluate the localization of phthalocyanine via red fluorescence [Fig.5].

<Fig. 5>

We performed flow cytometry analysis to accurately measure the rate of interaction between each macrocycle and the MCF7 cells. In this technique, cells were incubated with the photosensitizer and their fluorescence emissions were analyzed to determine the percentage of cells with increased emission intensities (due to the presence of the photosensitizer). We observed that symmetrical macrocycle **1** with protect hydroxyl groups showed higher cellular uptake than their unprotected counterpart, **2.** This result can be related to the highest polar character of macrocycle **2** and to the free hydroxyl group hydrogen bonds, which increase the macrocycle tendency to form aggregates and decrease their interaction with the target cells.

Almost all of the unsymmetrical phthalocyanines synthesized interacted well with the target cells. The results for unsymmetrical macrocycles (**4**), (**6**) and (**10**), with unprotected hydroxyl groups, indicated greater interaction with the target cells compared with their respective counterpart with protected hydroxyl groups (compounds (**3**), (**5**) and (**9**), respectively). The compounds (**7**) and (**8**) presented similar results of cell interaction. These observations indicate the importance of the hydrophobic/hydrophilic balance in the

interaction with the target cells. Further analyses are underway to understand the influence of these groups in each structure and to model their behavior and interactions with target cells.

4. Conclusions

A series of unsymmetrical phthalocyanines bearing polyhydroxylated moieties were synthesized and characterized and then investigated as photosensitizers in photodynamic therapy. The compounds have different tendencies to form aggregates in polar media, according to the number of attached moieties.

In vitro results showed that the presence of different proportions of hydroxylated moieties in the phthalocyanine macrocycle promoted different balances of hydrophilic and hydrophobic characteristics, which promoted distinct interactions with the target cells. These *in vitro* findings indicate these molecules offer good potential as photosensitizers in photodynamic therapy.

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Figure Captions

Fig. 1. The route of new phthalocyanines derivatives

Fig 2. ^{1}H NMR (500 MHz) spectrum of phthalocyanine **3** in $CDCl_3$.

Fig 3. UV-vis spectra of all phthalocyanine derivatives synthesized **1** (A), **3** (B) and **7** (C) in different concentrations (in DMSO).

Fig. 4. UV-vis spectra of phthalocyanines **5** (left) and **6** (right) in different proportions of H_2O and DMSO (each one at same concentration in all analysis, 7.6 $\mu mol.L^{-1}$).

Fig.5. Fluorescence images of the interaction between phthalocyanine **4** (45.0 µM final concentration) and MCF7 cells (grown on chamber slides at a density of 4 x 10^4 cells/cm^2 and treated with 300 nM DAPI solution). The images were produced by 340 nm excitation, using a 580 nm emission filter. DAPI was used to following the studied phthalocyanine (red fluorescence) according to the nuclei position.

Figure 1

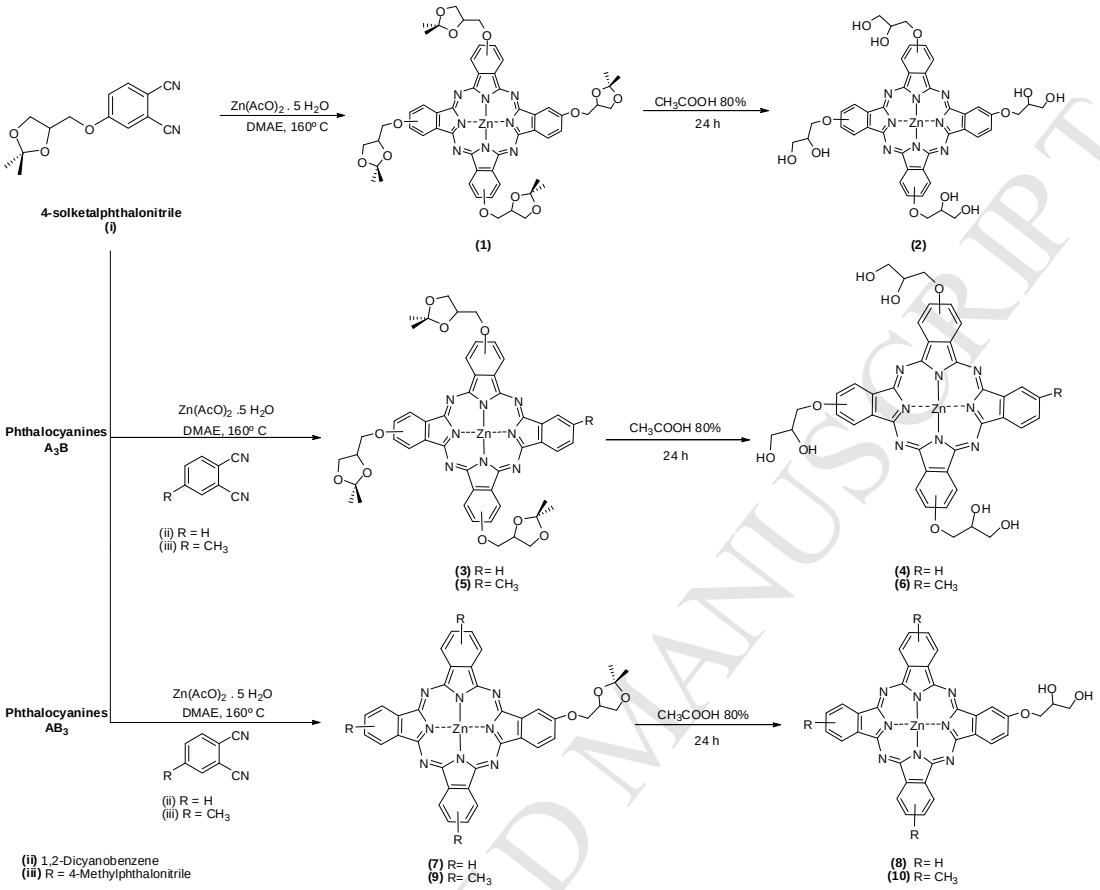


Figure 2

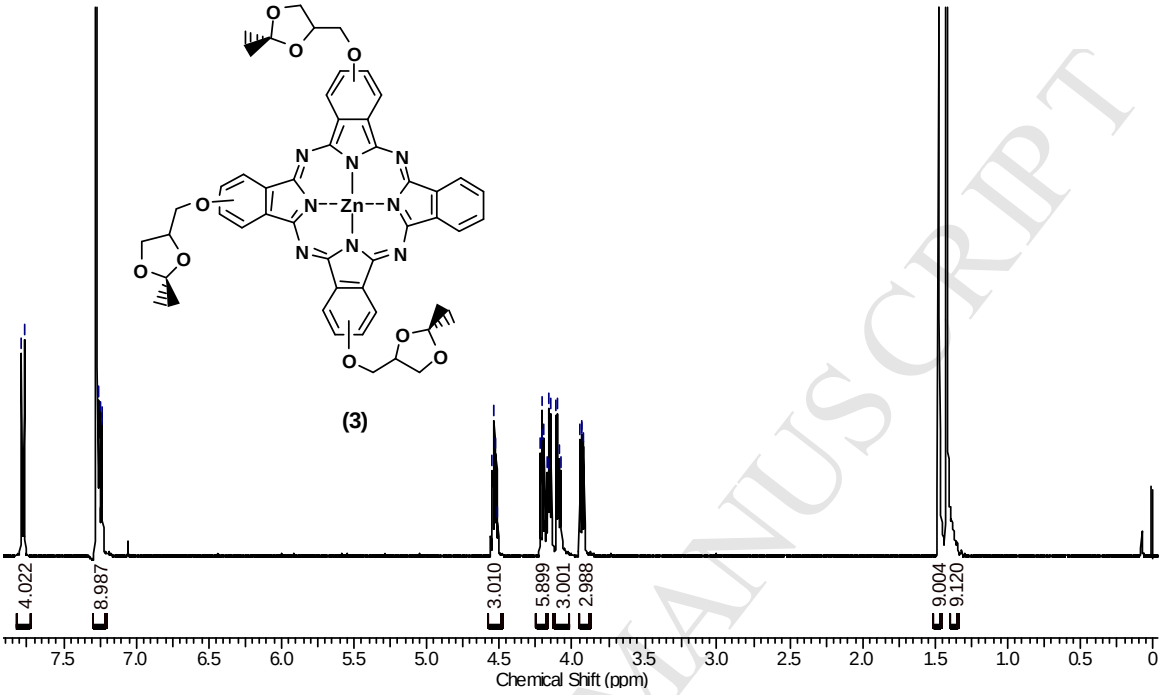


Figure 3

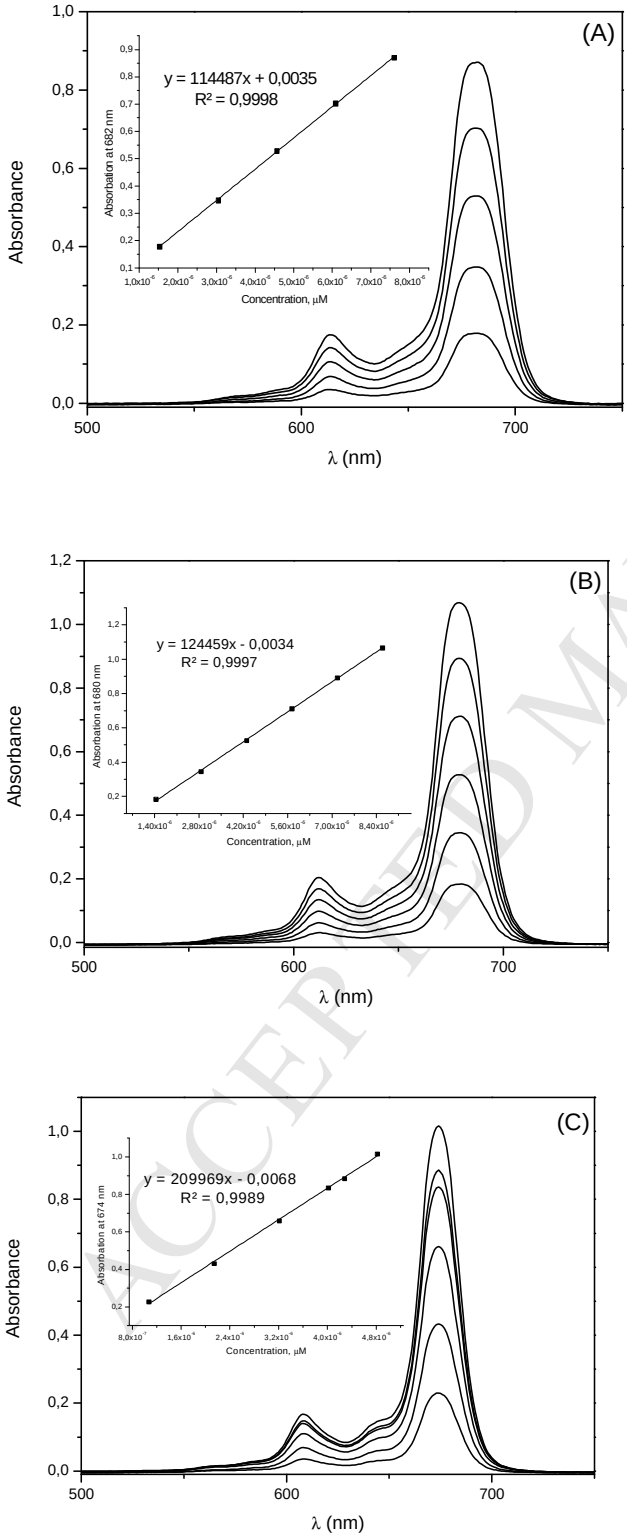


Figure 4

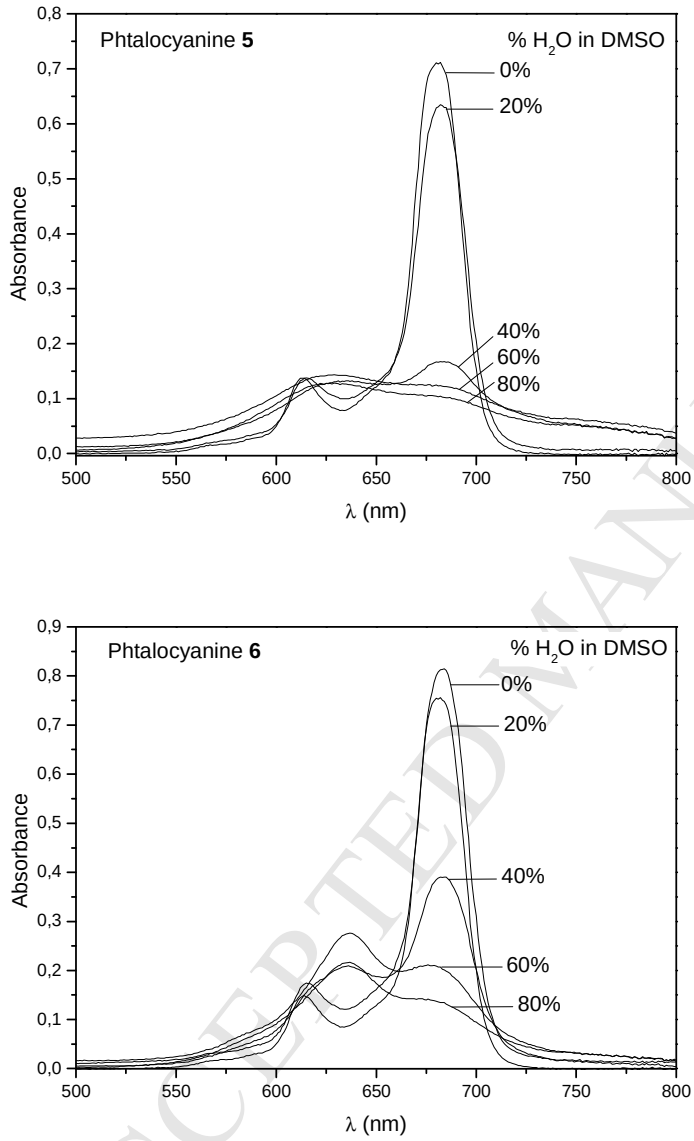


Figure 5

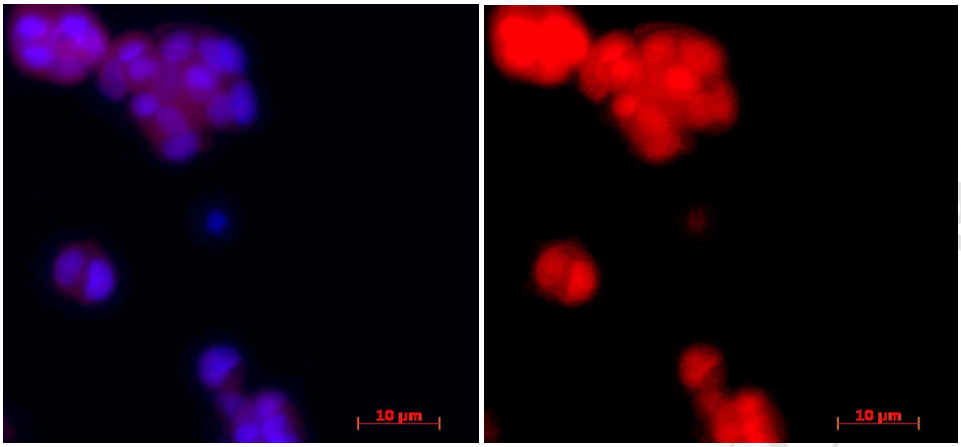
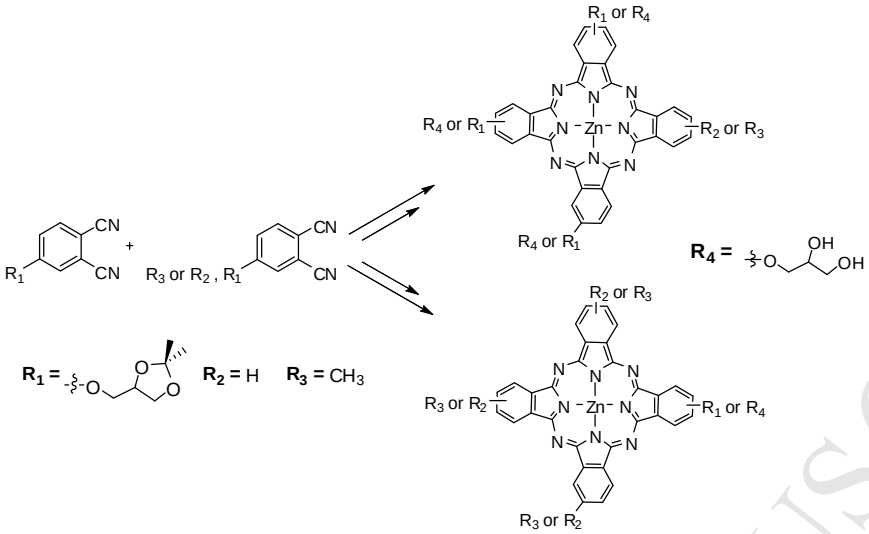


Table 1: Values for the absorbance maximum and quantum yield of singlet oxygen and fluorescence for phthalocyanines **1-10**. All data were recorded in DMSO.

Pc	UV-Vis λ_{max} (nm)	log ε	Log P	Φ_F	Φ_Δ	Cell interaction (%)*
1	358, 613, 682	4.72, 4.36, 5.05	1,23 ± 0,06	0.13	0.72	80 ± 1,2
2	358, 614, 682	4.89, 4.54, 5.23	0,58 ± 0,07	0.13	0.65	0
3	356, 612, 680	4.71, 4.38, 5.09	1,48 ± 0,08	0.13	0.67	62 ± 0,1
4	357, 612, 680	4.79, 4.46, 5.17	1,19 ± 0,08	0.13	0.44	84 ± 0,9
5	356, 613, 681	4.50, 4.16, 4.87	1,58 ± 0,07	0.13	0.71	0
6	356, 613, 681	4.66, 4.32, 5.03	1,46 ± 0,06	0.13	0.56	52 ± 0,2
7	350, 608, 674	4.79, 4.55, 5.32	1,47 ± 0,08	0.15	0.55	52 ± 0,1
8	354, 612, 679	4.76, 4.45, 5.18	1,35 ± 0,08	0.14	0.49	48 ± 0,6
9	353, 611, 679	4.53, 4.22, 5.00	1,67 ± 0,09	0.14	0.72	0
10	353, 612, 680	4.62, 4.31, 5.06	1,61 ± 0,08	0.14	0.62	81 ± 1,8

* The percentage of phthalocyanines entrance was determined quantitatively in MCF-7 cells, seeded in 12-well plates, incubated with 45.0 µM phthalocyanines for 1h, and performing the flow cytometric analysis.

Graphical abstract



Research Highlights

1. The aggregation and water solubility of the phthalocyanines derivatives is related to the proportion of free hydroxyl groups present in the structure.

2. The interaction between MCF7 mammary cell and the phthalocyanine derivatives in aqueous media changes according to the hydrophobic to hydrophilic balance in the macrocycle structure.

3. Unsymmetrical phthalocyanines with unprotected hydroxyl derivatives had a greater tendency to aggregate in aqueous media but higher interaction with MCF7 mammary cells.