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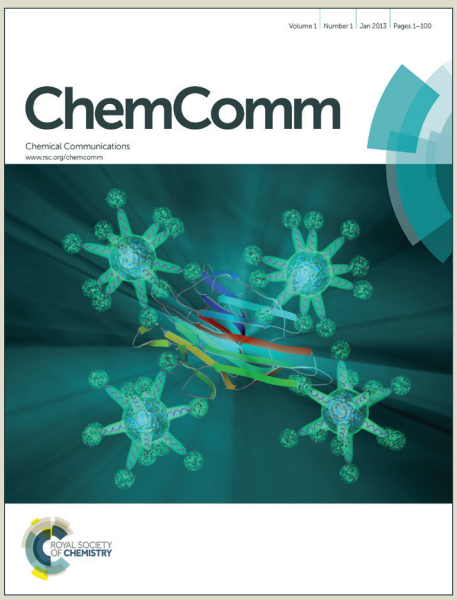

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Amphiphilic phthalocyanine-cyclodextrin conjugates for cancer photodynamic therapy

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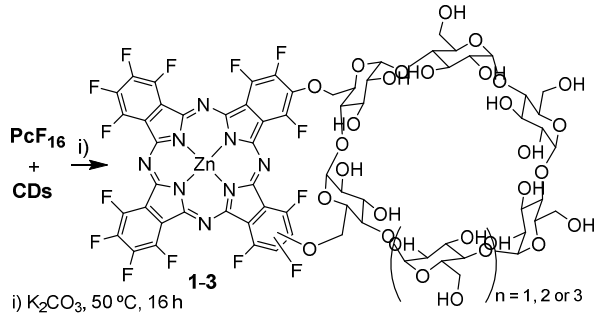
Three phthalocyanines (Pcs) conjugated with α-, β- and γ-cyclodextrins (CDs) were prepared and their application as photosensitizer (PS) agents assessed by photophysical, photochemical and *in vitro* photobiological studies. The photoactivity of Pc-α-CD and Pc-γ-CD ensure their potential as PDT drugs against UM-UC-3 human bladder cancer cells.

Phthalocyanines (Pcs) and cyclodextrins (CDs) have been intensively studied due to their applications in many scientific areas, namely in medicinal and supramolecular chemistry.[1-5] Pcs are well-known aromatic macrocycles with excellent photophysical properties to be used as photosensitizers (PSs) in photodynamic therapy (PDT) for the treatment of cancer.[6,7] This therapy combines visible light, molecular oxygen and a PS able to generate reactive oxygen species (ROS), like singlet oxygen ($^1O_2$), which can induce cell death pathways resulting in tumour tissues destruction. Pcs, besides their high efficiency in generating $^1O_2$, have the advantage to absorb light in the red and near-infrared regions.[7] The conjugation of Pcs with biochemical motifs is of utmost importance in the development of promising PSs, since it improves their solubility in water and are able to act as Pcs carriers, delivering them into cancer cells. CDs offer unique features towards this goal allowing the synthesis of compounds perfectly defined with specific structural characteristics. The most common natural CDs are the α-, β- and γ-CDs, constituted respectively by six (α-CD), seven (β-CD) and eight (γ-CD) glucopyranose units, bound *via* α-1,4-glycosidic linkages. Moreover, the conjugation of PSs with these non-toxic molecules can improve their amphiphilicity, biocompatibility and availability at the surface of cancer cell membranes.[8,9] In spite of the high potential of CDs as PS carriers, conjugates involving covalently linked Pcs and CDs are rare and only few works report their possible application as new PDT agents.[2,3,10-12] Aiming the development of tumor-targeting PSs, herein we report the preparation, characterization and *in vitro* evaluation of photodynamic efficacy of the new phthalocyanine-cyclodextrin (Pc-CD) conjugates **1-3** (Scheme 1). The effects of different CDs (α-, β- and γ-CDs) in the solubility of the Pc core, $^1O_2$ production, photostability, ability to interact with human serum albumin (HSA) and phototoxicity against UM-UC-3 human bladder cancer cells were studied.



1, **Pc-α-CD** (n=1); **2**, **Pc-β-CD** (n=2); **3**, **Pc-γ-CD** (n=3)

Scheme 1

Considering the remarkable photo-chemical and -physical properties of Pcs and the excellent features of CDs to act as Pc carrier, we envisaged a simple access to obtain amphiphilic Pc-CD conjugates *via* post-modification of the commercial available **PcF_{16}**. **Pc-α-CD 1**, **Pc-β-CD 2** and **Pc-γ-CD 3** were synthesized by nucleophilic substitution of two fluorine atoms of **PcF_{16}** (a single substitution of a β-fluoro atom in one of the isoindole unit followed by a second attack in one of the adjacent isoindole units) using, respectively, cyclo-maltohexaose (α-CD), cyclo-maltoheptaose (β-CD) and cyclo-maltooctaose (γ-CD) - scheme 1. A nucleophilic substitution in the same isoindole unit of the Pc is improbable due to the structural hindrance. It is noteworthy that the hydrophilic properties of the CDs[13] improve the water solubility of this Pc and proves to be an exceptional methodology[14] to prepare the water-soluble Pc conjugates **1-3**. The reactions between **PcF_{16}** and the adequate equimolar quantity of CDs (α-, β-, and γ-CD) were performed in 10 mL of dimethyl sulfoxide (DMSO) in the presence of excess potassium carbonate (8 equiv.) at 50 ºC. These reactions were finished after stirring for 16 h and precipitated in chloroform. The corresponding Pc-CD conjugates were purified by silica gel and reverse phase column chromatography, using a gradient of tetrahydrofuran/water as eluent; and finally by molecular exclusion column

chromatography using DMF as solvent. The purified Pc-CDs were reprecipitated in chloroform. The structures of dyads **1-3** were confirmed by UV–Vis spectroscopy, ^{19}F NMR and MALDI-TOF mass spectrometry. The degree of homogeneity of the sample was assayed by HPLC analysis (see Supporting Information, SI).

Amongst the techniques used for the characterization of the Pc-CDs **1-3**, the most appropriate one was MALDI-TOF-MS, which is very useful for sequencing and structurally analysing saccharide and oligosaccharide derivatives,[15,16] namely CDs[17] and glycophthalocyanines.[18] Moreover, the study by MS/MS can also be an excellent and valuable tool to assess structural characterization. The MALDI-MS spectra of the Pc-CDs showed the molecular ion peak $[M+Na]^+$ of the Pc-CDs at *m/z* 1821, 1981 and 2143 for **Pc-α-CD**, **Pc-β-CD** and **Pc-γ-CD**, respectively. These $[M+Na]^+$ ions confirmed the nucleophilic substitution of two β-fluorine atoms of Pc by the corresponding α-, β-, γ-CDs, accompanied by elimination of two molecules of hydrofluoric acid.[19] The formation of $[M+Na]^+$ ions are typical of glycoderivatives,[20-22] and it was previously observed in other glycophthalocyanines.[18] MALDI-MS/MS spectra were acquired to confirm the structural assignment of each $[M+Na]^+$ ion of **1-3**, respectively, and the spectra are displayed in figures SI 17-22 and in table SI 2. Based on the mass spectra results (see also details in SI mass discussion) and considering that the second substitution reaction of the CD in the same isoindol unit of the Pc is less probable, and even less probable in the opposite isoindol ring, we considered that the most plausible regioisomers must be provided from nucleophilic substitution reactions in adjacent isoindol units of the Pc, as depicted in scheme 1.

The spectroscopic properties of the Pc-CDs **1-3** are summarized in table 1. In DMSO and in PBS (phosphate buffered saline) , the compounds exhibited very similar absorption spectra with strong Q absorption bands at the red visible region (694-699 nm, figures 1A and SI 1). The Pc-CDs in DMSO followed the Lambert-Beer's law (Figure SI 2), suggesting that solubility was not compromised for concentrations ranging from 0 to 50 μM. Upon excitation at 610 nm, the compounds in DMSO showed the same emission behaviour with a emission band in the red spectral region at 698-705 nm (Figure 1B) and fluorescence quantum yields (Φ_F) of 0.10-0.28, described in table 1, relative to the unsubstituted phthalocyaninatozinc(II) (**Pc**, Φ_F = 0.20 in DMSO).[23] The fluorescence quantum yields decrease in the order to **1** > **2** > **3**.

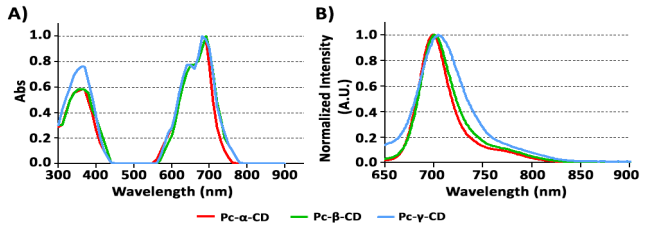


Fig. 1. Normalized electronic A) absorption and B) emission ($\lambda_{exc.}$ = 610 nm) spectra of **1-3** in DMSO.

The emission spectra of **Pc-α-CD** and **Pc-γ-CD** demonstrated similar shapes with maximum emission bands at 688 and 682 nm, respectively, in PBS (Figure SI 1). On the other hand, **Pc-β-CD** demonstrated a maximum emission band at 717 nm also in PBS (Figure SI 1).

The water solubility of the new PSs is a critical parameter for the photodynamic effect, as low solubility compromises their bio-distribution, uptake by the cells and ROS production. The solubility of Pc-CDs **1-3** in PBS was investigated for concentrations ranging from 0 to 50 μM (with <1% v/v DMSO, Figure SI 3), in close resemblance conditions to those used in the *in vitro* PDT assays.

Table 1. Photophysical data of Pc-CDs **1-3**. Binding constant (K_a) and number of binding sites (n) of **1-3** to HSA.

Pc-CD	Q band λ_{max} (nm)	$\lambda_{emission}$ (nm)[a]	Φ_F[b]	HSA interaction K_a (M^{-1})	n
1	694	698	0.28	$3.0x10^5$	1.2
2	696	700	0.18	$1.1 x10^2$	1.3
3	699	705	0.10	$7.8x10^5$	0.9

[a]excited at 610 nm;[b]**ZnPc** in DMSO as reference (Φ_F = 0.20).[23]

The solubility of the Pc-CDs decreased in the order **3** ≈ **1** > **2** in PBS. The Q absorption bands of **Pc-α-CD** and **Pc-γ-CD** were significantly broadened, but still followed the Lambert-Beer's law, suggesting that solubility was not compromised under these conditions. However, the absorption spectra of **Pc-β-CD** indicated that the solubility of this compound is lower in PBS (Figure SI 3) than in DMSO (Figure SI 2). The self-aggregation of **Pc-β-CD** can be a possible explanation for its lower water solubility when compared with **Pc-α-CD** and **Pc-γ-CD**. In fact, the self-aggregation of drug-loaded CDs in water is a well-known phenomenon,[24] which can be caused by the self-aggregation tendency of β-CD native structure,[25] since its molecular dimensions are optimal for the formation of an intramolecular hydrogen bond within the CD molecule, preventing its hydrogen bond formation with surrounding water molecules and reducing its solubility.[25] From our results, we hypothesize that β-CD is less soluble themselves, which makes the conjugate with Pc with low water solubility.

Considering the potential application of Pc-CDs **1-3** as new PSs, their photostability and ability to generate $^1O_2$ were determined. The photostability of Pc-CDs **1-3** was determined in PBS (with <1% v/v DMSO) at different irradiation times, by monitoring the absorption Q band intensity decrease under white (400–800 nm) and red (620–750 nm) lights, both at a rate of 150 mW.cm^{-2}. The Pc-CD dyads **1-3** exhibited similar photostability when compared to **Pc** over the investigated irradiation period (40 min; Table SI 1). These results demonstrated that the derivatisation of Pc with α-, β- and γ-CDs did not compromise their photostability.

The ability of Pc-CD conjugates **1-3** to generate $^1O_2$ was evaluated in DMF/H_2O (9:1 v/v) and in DMSO. On these two solvents, the three conjugates strictly followed the Lambert-Beer's law between 0 to 50 μM (Figures SI 2 and 4), suggesting that the solubility of Pc-CD conjugates was not compromised during the study. For the determination of $^1O_2$ generation, it was performed the steady-state method using 1,3-diphenylisobenzofuran (DPBF) as the $^1O_2$ acceptor and **Pc** as the $^1O_2$ generator reference compound.[26] The decrease in the absorption of DPBF (monitored at 415 nm) was higher in the presence of all Pc-CDs (Figures SI 5 and 6) with the conjugates **1** and **3** showing similar ability to photooxidize DPBF when compared to **Pc**. These results demonstrated that **Pc** moiety did not lose its sensitizing properties after binding with α- and γ-CDs. **Pc-β-CD** demonstrated to be a much less efficient $^1O_2$ generator, which could be partially explained by its low solubility in aqueous media like PBS (Figure SI 3).

Knowing that human serum albumin (HSA) is able to bind anticancer drugs and deliver them to the target organs, the interaction of Pc-CDs **1-3** with the abundant plasma protein HSA was studied by emission quenching of tryptophan residues in HSA solutions, after addition of Pc-CDs at increasing concentrations (0-10 μM in PBS with <1% v/v DMSO).[26] The addition of Pc-CDs to HSA led to emission quenching of tryptophan residues (Figure SI 7), which was lower for the

compound **2** when compared with compounds **1** and **3**. The binding constant (K_a) and the number of binding sites (n) of compounds **1-3** were determined as described in the literature[26] and compared with the ones obtained for **ZnPc** ($K_a = 7.3 \times 10^3$ M^{-1} and n = 0.8). The K_a values of the Pc-CDs decreased in the order **3** > **1** > **Pc** > **2** (Table 1). The number of binding sites indicates that there is only one binding site for the Pc-CDs closes to the tryptophan residues of HSA (Table 1). The values of K_a obtained for Pc-CDs **1** and **3** are in accordance to the ones obtained for Pcs glycodendritic conjugates.[26]

The *in vitro* photosensitizing efficiency of Pc-CDs **1-3** was performed in the human bladder cancer cell line (UM-UC-3) derived from transitional cell carcinoma. The cytotoxicity of Pc-CDs **1-3** was investigated in the presence and absence of light using MTT colorimetric assay (Figures SI 11 and 12). Pc-CDs solutions **1-3** were non-toxic in the dark up to 10 μM and uptake time up to 4 h. For the PDT assays, the bladder cancer cells were incubated in darkness with Pc-CDs at different concentrations (0-1 μM) and irradiated with a white (400–800 nm) or a red (620–750 nm) light source, both at a fluence rate of 50 $mW.cm^{-2}$. Variable irradiation times of 20 and 40 min were performed for giving a demonstration of irradiation-time dependence of cell killing. Compounds **1** and **3** demonstrated a phototoxic effect in a concentration- and irradiation time-dependent manner (Figure SI 12). The IC_{50} values are summarized in table 2, estimated from figure SI 12, defined as the Pc-CD concentration required to kill 50% of the UM-UC-3 bladder cancer cells. The data show that the cell photokilling is dependent of the Pc-CD, light source, and irradiation time. In case of the white light source, only a narrow region of the light was absorbed by the compounds to initiate the photodynamic action. Hence, the IC_{50} values were lower when it was used the red light source. The data showed interesting differences between the photoactivity of Pc-CDs that differ only in the CD nature. Compounds **1** and **3** are more phototoxic comparatively with **2**, showing IC_{50} values in the range of 26-87 and 330-460 nM when cells are irradiated for 20 and 40 min with red and white lights, respectively. These IC_{50} values of Pc-CDs **1** and **3** were similar with the obtained for β-CD-conjugated silicon(IV) Pcs against human colorectal carcinoma and human hepatocarcinoma cells.[3]

Table 2 – IC_{50} values of Pc-CDs **1-3** against UM-UC-3 cancer cells.

	Pc-CD	Red light		White light	
		20 min	40 min	20 min	40 min
IC_{50} (nM)	**1**	41	33	460	380
	2	n/d	n/d	n/d	n/d
	3	87	26	430	330

IC_{50}: incubation concentration that inhibits the proliferation of cultures in 50%, after cells incubation with Pc-CD and irradiation; n/d: not determined.

The lower solubility and $^1O_2$ production of **2** could explain the absence of phototoxic effect on UM-UC-3 cells (Figure SI 12). Meanwhile, it is well-known that aggregation can deactivate the excited electronic states of PSs and cause further loss of photoreactivity.

To account for the difference in photocytotoxicity, the intracellular production of ROS of these Pc-CDs was evaluated immediately after PDT using the probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA).[27] It was found that all the Pc-CDs are able to generate ROS upon irradiation with white or red light; and the intracellular ROS production was higher for Pc-CD dyads **1** and **3** than for **2** (Figure SI 13), which is in good agreement with the trend observed for their photocytotoxicity (Table 2).

In addition to the cell viability studies, we also investigated the intracellular uptake of Pc-CDs **1-3** in bladder cancer cells by fluorescence microscopy (Figure 2). It is expected that CDs act as Pc carriers and that the hydrophilic (polar) CD exterior and lipophilic (nonpolar) Pc ring of Pc-CDs **1-3** resemble the structure of amphiphilic sensitizers. Uptake of these types of PSs by endocytosis has been previously reported.[5] Moreover, it has been reported that CDs can perturb the lipophilic membrane barrier, enhancing the uptake of Pcs in cancer cells.[5]

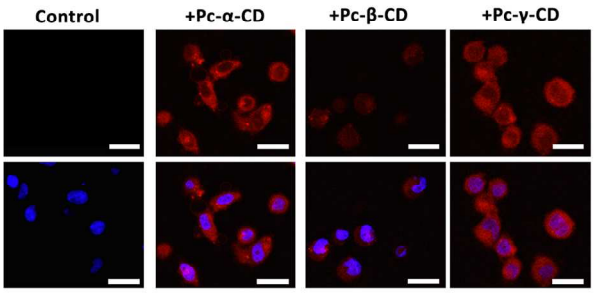


Fig. 2. Representative fluorescence images of UM-UC-3 bladder cancer cells incubated with Pc-CDs (red) in darkness and cell nucleus stained with DAPI (blue). *Scale bars* 20 μm.

The results obtained by fluorescence confocal microscopy revealed that incubation of UM-UC-3 cancer cells with the three Pc-CDs at 1 μM for 3 h (in the dark) led to cell incorporation of all compounds resulting in intracellular fluorescence. There were marked differences in the cellular uptake of **2** when compared with **1** and **3**, which might be due to its overall solubility and tendency to aggregate. While **2** is poorly taken up by the cells, **1** and **3** are accumulated inside the cells to a much higher extent. All these results suggest that the higher phototoxicity of compounds **1** and **3** can be attributed to their higher cellular uptake and efficiency in generating intracellular ROS.

Conclusions

We have prepared and characterized three Pc-CD conjugates (**Pc-α-CD**, **Pc-β-CD**, and **Pc-γ-CD**) and investigated their PS capabilities on UM-UC-3 bladder cancer cell line. The new Pc-CD conjugates were structurally well characterized by MALDI MS/MS tandem mass spectrometry, in which the observed fragmentation pathways were therefore relevant for the identification of such glycophthalocyanine derivatives. The new **Pc-α-CD** and **Pc-γ-CD** exhibited much higher water-solubility, $^1O_2$ production and intracellular ROS generation than **Pc-β-CD**, with consequently much higher UM-UC-3 bladder cancer cell line phototoxicity by the first two Pc-CDs and almost none toxicity by the **Pc-β-CD**. The promising photoactivity of **Pc-α-CD** and **Pc-γ-CD** ensure their potential as strong PDT drugs and it opens the possibility to explore these and many other bioconjugates of $\mathbf{PcF_{16}}$ in different directions.

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Notes and references

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† Electronic Supplementary Information (ESI) available: synthesis, mass, photo-physical and -biological studies and spectral data. See DOI: 10.1039/c000000x/

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