Supporting Information

Amphiphilic phthalocyanine-cyclodextrin conjugates for cancer photodynamic therapy

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1 Experimental section

1.1 General methods

^{1}H and ^{19}F NMR spectra were recorded on a *Bruker Avance-300* spectrometer at 300.13 and 282.38 MHz and at 60 ºC, respectively. Tetramethylsilane was used as internal reference. Absorption and fluorescence spectra were recorded using a *Shimadzu UV-2501-PC* and *FluoroMax3 (Horiba JovinYvon)*, respectively. Analytical TLC was carried out on precoated silica gel sheets (Merck, 60, 0.2 mm). Column chromatography was carried out over silica gel (Merck, 63–200 mesh). Reverse phase column chromatography was carried out over Sep-Pak®Vac 35cc (10 g) tC18 Cartridges and molecular exclusion column chromatography was carried out over Bio-beads™ S-X1 Beads (200-400 Mesh, 100 g), Bio-Rad Laboratories, Inc.

The HPLC chromatograms were recorded on a Merck Hitachi L620A coupled to a detector Merck Hitachi, L4250 UV/Vis detector, set at 690 nm.

MALDI-MS and MALDI-MS/MS mass spectra were acquired using a MALDI-TOF/TOF Applied Biosystems 4800 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA) instrument equipped with a nitrogen laser emitting at 337 nm. Prior to MALDI-MS analysis, 4 µL of matrix, dithranol (10 $mg.mL^{-1}$ in methanol / 0.1% TFA) were mixed with 2 µL of Pc-CD solution in methanol/ (≈10 $\mu g.mL^{-1}$), and 1 µL of this mixture was deposited on the MALDI plate and let to dry. MALDI-MS spectra were acquired in the positive ion reflector mode using delayed extraction in the mass range between 600 and 4500 Da with *ca*. 1500 laser shots. For the following acquisition of tandem mass spectra (MS/MS) a collision energy of 2 keV was used to induce fragmentation, and air was used as collision gas.

1.2 Synthesis and characterization of new compounds

General procedure for the preparation of the Pc-CD derivatives 1-3:

Hexadecafluorophthalocyaninatozinc(II) **PcF_{16}** (50 mg) and 1 equivalent of the CD derivative (α-, β- and γ-CD) reacted in dry DMSO (10 mL), in presence of an excess of K_2CO_3, for 16 h hat 50 ºC until no starting **PcF_{16}** could be seen by TLC. After that the reaction mixture was precipitated with chloroform. The crude mixture was filtrated and redissolved in THF/H_2O (6/4), and the products firstly separated by silica gel column chromatography using a gradient of THF/H_2O. Later, the corresponding product fraction was purified by reverse phase column chromatography, using again a gradient of THF/H_2O as eluent, and by molecular exclusion column chromatography using DMF as solvent. The

desired products were then directly precipitated from THF/H_2O. Typical yields were over 63%. The structures of dyads **1-3** were confirmed by NMR spectroscopy, UV–Vis and MALDI-TOF-MS.

1.2.1 Phthalocyanine-α-cyclodextrin dyad (Pc-α-CD, 1)

In a 25 mL round-bottom flask was dissolved **PcF_{16}** (50.0 mg, 0.058 mmol) and α-CD (57.7 mg, 0.059 mmol, 1.0 equiv.) in DMSO (10 mL), and then added K_2CO_3 (62.5 mg, 0.45 mmol, 7.8 equiv.). The reaction mixture was stirred for 16 h at 50ºC. After the workup and the purification described above the product **Pc-α-CD** was crystalized from THF/H_2O and obtained in 73% (76.8 mg) yield. ^{19}F NMR (282 MHz, DMSO-d_6): δ -178.86 to -175.51 (m, 8F, 8-α-F), -166.98 to -164.12 (m, 6F, 6-β-F). UV-Vis (DMSO), $\lambda_{max.}$ (log ε): 694 (3.67). MALDI-TOF-MS: *m/z* 1821 $[M+Na]^+$.

1.2.2 Phthalocyanine-β-cyclodextrin dyad (Pc-β-CD, 2)

In a 25 mL round-bottom flask was dissolved **PcF_{16}** (52.0 mg, 0.059 mmol) and β-CD (67.0 mg, 0.059 mmol, 1.0 equiv.) in DMSO (10 mL), and then added K_2CO_3 (68.5 mg, 0.50 mmol, 8.4 equiv.). The reaction mixture was stirred for 16 h at 50ºC. After the workup and the purification described above the product was crystalized from THF/H_2O and obtained in 66% (77.2 mg) yield. ^{19}F NMR (DMSO-d_6, 282 MHz): δ -178.30 to -175.26 (m, 8F, 8-α-F), -166.78 to -164.15 (m, 6F, 6-β-F). UV-Vis (DMSO), $\lambda_{max.}$ (log ε): 696 (3.03). MALDI-TOF-MS: *m/z* 1981 $[M+Na]^+$.

1.2.3 Phthalocyanine-γ-cyclodextrindyad(Pc-γ-CD, 3)

In a 25 mL round-bottom flask was dissolved **PcF_{16}** (50.8 mg, 0.058 mmol) and γ-CD (77.4 mg, 0.060 mmol, 1.0 equiv.) in DMSO (10 mL), and then added K_2CO_3 (66.0 mg, 0.48 mmol, 8.2equiv). The reaction mixture was stirred for 16 h at 50ºC. After the workup and the purification described above the product was crystalized from THF/H_2O and obtained in 63% (78.4 mg) yield. ^{19}F NMR (DMSO-d_6, 282 MHz): δ -179.92 to -176.90 (m, 8F, 8-α-F), -167.16 to -165.02 (m, 6F, 6-β-F). UV-Vis (DMSO), $\lambda_{max.}$ (log ε): 699 (3.91). MALDI-TOF-MS: *m/z* 2143 $[M+Na]^+$.

1.3 HPLC analysis of Pc-CD conjugates

The degree of homogeneity of the Pc-CD conjugates was analyzed by HPLC using the following experimental conditions:

Solvent A: Water (33%); **Solvent B**: Methanol (20%); **Solvent C**: Tetrahydrofuran (47%)

Column: Waters Spherisorb C8, 54.6 × 250 mm

Flow: 0.5 mL/min; **Chart Speed**: 1.25 mm/min

The **Pc-β-CD** conjugate was found to elute as a reasonably sharp band at 20 min (uncorrected retention time) in a recently conditioned column (1/1 methanol, THF). Retention times drop down to about 18 min after a series of six injections.

At higher concentrations a small peak elutes at 30 min, which accounts for less than 4.5% of the area. Visible spectra were obtained for these peaks, using the built in facilities of the detector.

Under similar conditions the **Pc-α-CD** conjugate elutes at 15 min as a sharp band, with a small peak at 23 min, accounting for less than 2.5% of the area.

Best conditions for the elution of the **Pc-γ-CD** conjugate were as follows: water/methanol/THF (40/20/40) for 10 min, increasing to 0/20/80 during 5 min, with a 1.5 mL/min flow. The compound elutes at 17 min as a broad band.

1.4 Solubility assays

Absorption and fluorescence spectra of Pc-CDs **1-3** were recorded on a UV-2501 PC Shimadzu and FluoroMax3 spectrophotometers, respectively. Stock solutions of the conjugates at a concentration of 20 mM were prepared in DMSO and stored in the dark at room temperature (rt). The working solutions were freshly prepared prior to use by diluting the stock solutions in DMSO (Figure SI 2), DMF/H_2O (9:1 v/v, Figure SI 4) or phosphate buffered saline (PBS) (10 mM NaH_2PO_4, 70 mM Na_2HPO_4 and 145 mMNaCl at pH 7.6, Figure SI 3) with the concentration of DMSO being always below to 1% (v/v).

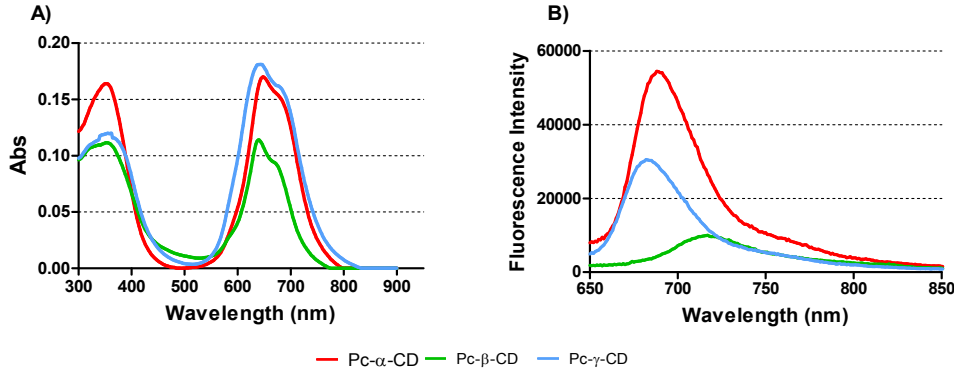


Figure SI 1 – A) absorption and B) Emission ($\lambda_{exc.}$ = 610 nm) spectra of **Pc-α-CD**, **Pc-β-CD** and **Pc-γ-CD** at 5 μM in PBS.

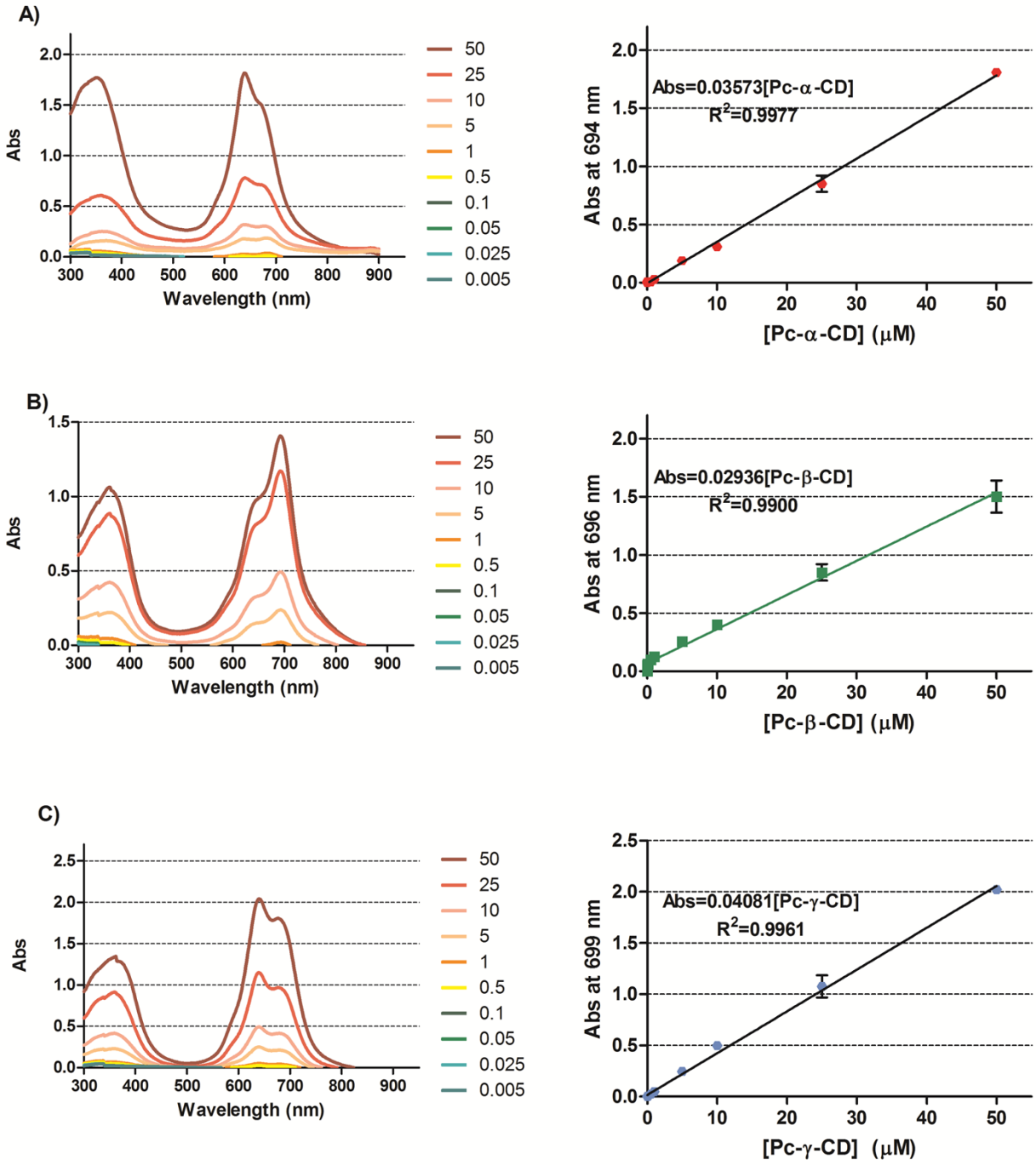


Figure SI 2 – UV-Visible spectra of A) **Pc-α-CD1**, B) **Pc-β-CD2** and C) **Pc-γ-CD3** in DMSO at different concentrations 0 to 50 μM. The inset of each spectrum plots the Q-band absorbance *vs* the concentration of the Pc-CD in DMSO, and the line represents the best-fitted straight line.

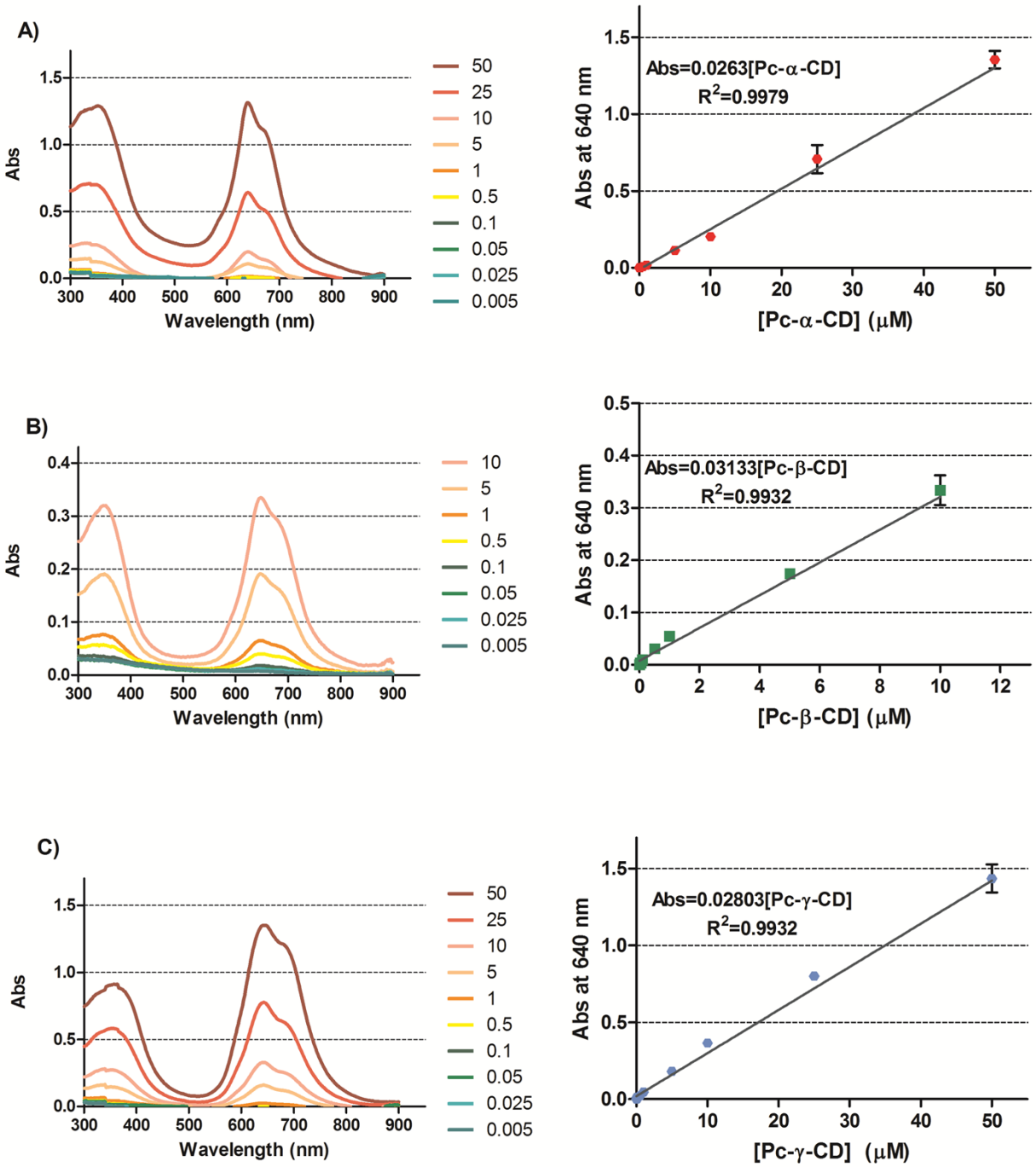


Figure SI 3 – UV-Visible spectra of A) **Pc-α-CD 1**, B) **Pc-β-CD 2** and C)**Pc-γ-CD 3** in PBS (<1% v/v DMSO) at different concentrations (**Pc-α-CD 1** and **Pc-γ-CD 3**: 0 to 50 μM, **Pc-β-CD 2**: 0 to 10 μM). The inset of each spectrum plots the Q-band absorbance *vs* the concentration of the Pc-CD in PBS, and the line represents the best-fitted straight line.

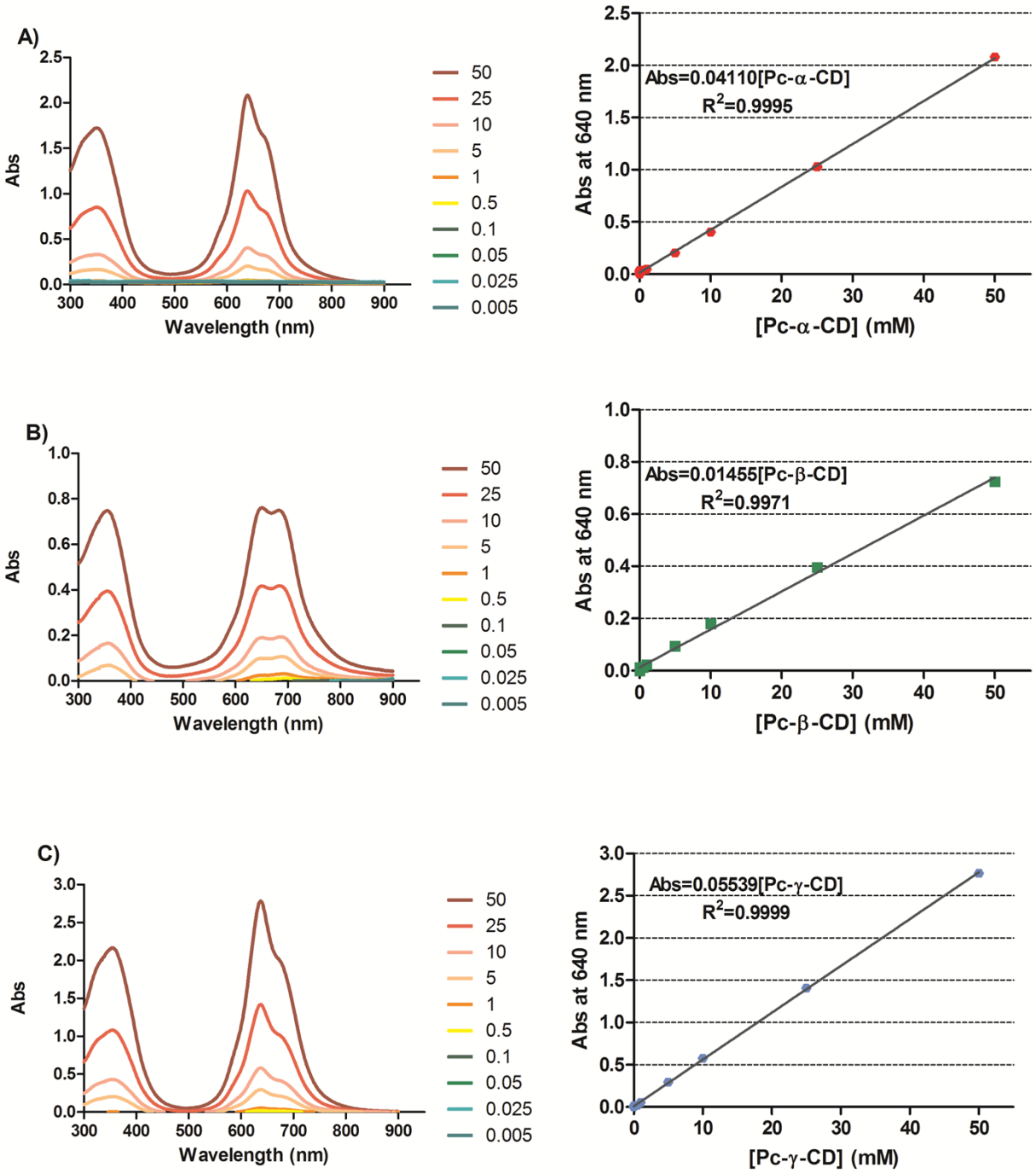


Figure SI 4– UV-Visible spectra of A) **Pc-α-CD 1**, B) **Pc-β-CD 2** and C) **Pc-γ-CD 3** in DMF/H_2O (9:1 v/v) at different concentrations 0 to 50 μM. The inset of each spectrum plots the Q-band absorbance *vs* the concentration of the Pc-CD in DMF/H_2O (9:1 v/v), and the line represents the best-fitted straight line.

1.5 Fluorescence assays

The emission spectra of Pc-CDs **1-3** were measured in DMSO in 1 cm × 1 cm quartz optical cells under normal air conditions on a computer controlled FluoroMax3 Horiba JobinYvon spectrofluorimeter. The widths of both excitation and emission slits were set at 2.0 nm. The fluorescence quantum yields (Φ_F) of Pc-CDs were calculated in DMSO by comparison of the area below the corrected emission spectra (between 650 to 800 nm) using ZnPc as standard ($\lambda_{exc.}$ at 610 nm, $\Phi_f = 0.20$ in DMSO) (see reference *Nyokong et al.*). For that, the following equation was used:

$$\Phi_f^{sample} = \Phi_f^{standard} \frac{AUC^{sample}(1 - 10^{-Abs_{standard}})}{AUC^{standard}(1 - 10^{-Abs_{sample}})}$$

where *AUC* is the integrated area under the fluorescence curves for each sample and standard, and *Abs* is the absorbance of the samples and the standard at the excitation wavelength ($\lambda_{exc.}$ at 610 nm).

1.6 Photostability assays

For the photostability assays, solutions of Pc-CDs **1-3** at 1 µM were freshly prepared in PBS (with 0.5% v/v DMSO) and kept in the dark at room temperature. The irradiation experiments were performed in magnetically stirred cuvette solutions (with 2 mL of sample), over a period of 40 min with white (400-800 nm) and red light (620-750 nm) delivered by an illumination system (LC-122 LumaCare, London) equipped with a halogen/quartz 250 W lamp coupled to the selected interchangeable optic fiber probe (400-800 nm or 620-750 nm). The lights were delivered at a fluence rate of 150 mW.cm^{-2}, measured with an energy meter Coherent FieldMaxII-Top combined with a Coherent PowerSens PS19Q energy sensor. The absorbance at 640 nm was determined at 0, 1, 3, 4, 5, 10, 15, 20, 25, 30, and 40 min periods of time after irradiation. The results were expressed as follows:

$$Photostability\ (\%) = \frac{Abs\ at\ a\ given\ time\ of\ irradiation}{Abs\ before\ irradiation}$$

Table SI 1 –Photostability of 1 μM of ZnPc and Pc-CDs **1-3** in PBS, after irradiation with white (400-800 nm) and red light (620-750 nm) at a fluence rate of 150 mW.cm^{-2} for different periods of time (0-40 min).

Compounds	Light	Irradiationtime (min)										
		0	**1**	**3**	**4**	**5**	**10**	**15**	**20**	**25**	**30**	**40**
ZnPc[a]	**white**	100	99	98	97	97	96	94	93	92	91	90
	red	100	99	98	98	98	97	97	96	96	96	96
Pc-α-CD	**white**	100	99	98	98	98	97	97	96	95	95	95
	red	100	100	100	100	100	100	100	100	100	99	99
Pc-β-CD	**white**	100	97	95	95	94	94	94	94	94	94	94
	red	100	99	99	98	97	96	95	95	94	94	93
Pc-γ-CD	**white**	100	99	99	99	99	99	99	96	96	96	96
	red	100	99	99	99	99	99	99	99	99	98	98

[a]Thephotostability of ZnPc was determined in PBS buffer containing 20% of DMSO due to its low solubility in aqueous solution. The results are presented in percentage calculated by the ratio of residual absorbance at 640 nm at different periods of time and absorbance before irradiation.

1.7 Singlet oxygen production

For the determination of singlet oxygen production, solutions containing DPBF (33 μM) with or without Pc-CDs or ZnPc at 0.33 μM were prepared in DMF/H_2O (9:1 v/v, Figure SI 4) or in DMSO (Figure SI 5) in a quartz cuvette. The solutions were irradiated at room temperature and under gentle magnetic stirring, with a LEDs array system emitting red light (λ > 600 nm) at a fluence rate of 10 mW.cm^{-2}. The breakdown of DPBF was monitored by measuring the decrease in absorbance at 415 nm at pre-established irradiation intervals. The results were expressed by plotting the DPBF depletion against the irradiation time. The depletion of DPBF was calculated as follows: $DPBF\ depletion = \frac{Abs_t}{Abs_0}$.

Abs_0 and Abs_t are the absorbance values at 415 nm before and after irradiation, respectively.

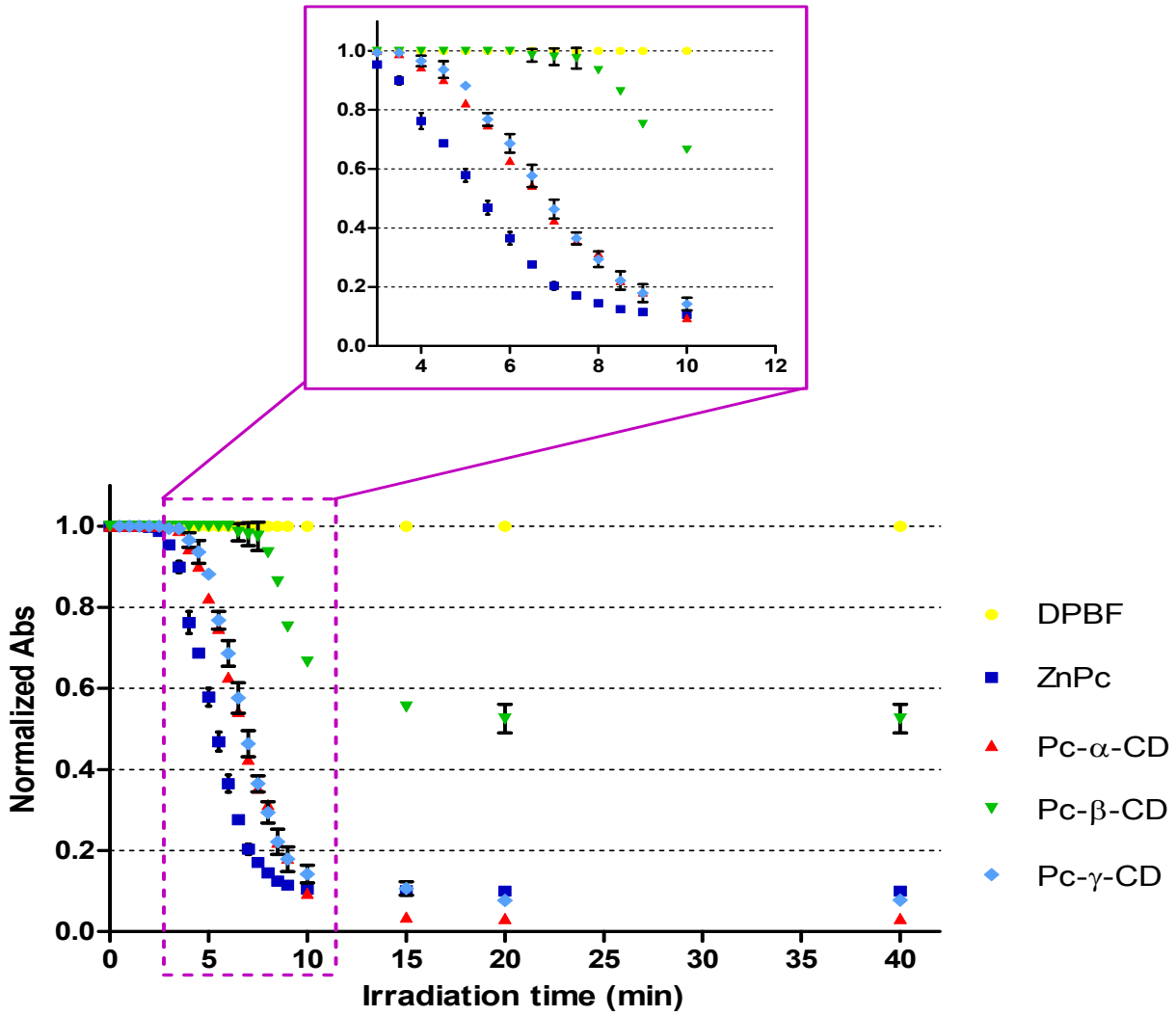


Figure SI 5 – Photo-oxidation of DPBF (33 µM) in DMF/H_2O (9:1) with or without **Pc-α-CD 1**, **Pc-β-CD 2**, **Pc-γ-CD 3** or ZnPc at 0.33 µM, after irradiation with a LEDs array system emitting red light at a potency of 10 mW.cm^{-2}. The DPBF absorbance was recorded at 415 nm.

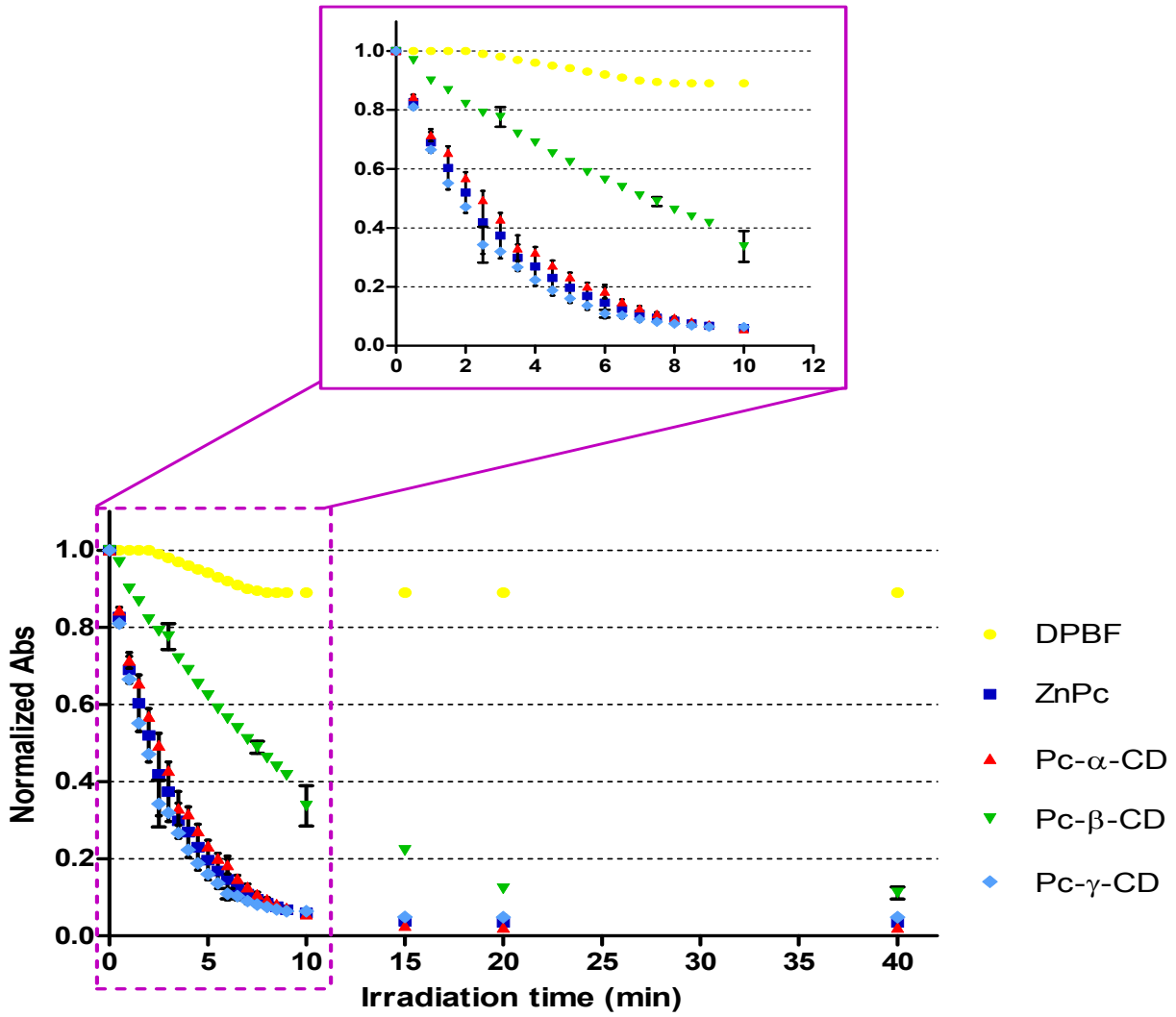


Figure SI 6 – Photo-oxidation of DPBF (33 µM) in DMSO with or without **Pc-α-CD 1**, **Pc-β-CD 2**, **Pc-γ-CD 3** or ZnPc at 0.33 µM, after irradiation with a LEDs array system emitting red light at a potency of 10 mW.cm^{-2}. The DPBF absorbance was recorded at 415 nm.

1.8 Human serum albumin (HSA) interaction assays

For the determination of Pc-CDs interaction with HSA, 2 mL of HSA solution was titrated with increasing concentrations of Pc-CDs, keeping always the final amount of DMSO below 1% (v/v). The emission spectra of the HSA´s tryptophan residues were acquired for the wavelength range between 300-450 nm upon excitation at 280 nm. The excitation and emission slits width were set at 2.0 nm. The emission quenching curves were obtained by plotting the tryptophan residues quenching (in percentage) against conjugates concentration. The tryptophan residues quenching (in percentage) was calculated, as follows:

$$Trypthophan\ residues\ quenching\ (\%) = \frac{(F_0 - F)}{F_0} \times 100$$

The K_a and n values were determined by plotting the $log((F_0-F)/F)$ against $log(Pc\text{-}CD\ concentration)$, giving a linear plot, where $log(K_a)$ and $log(n)$ are the ordinate at the origin and slope, respectively.

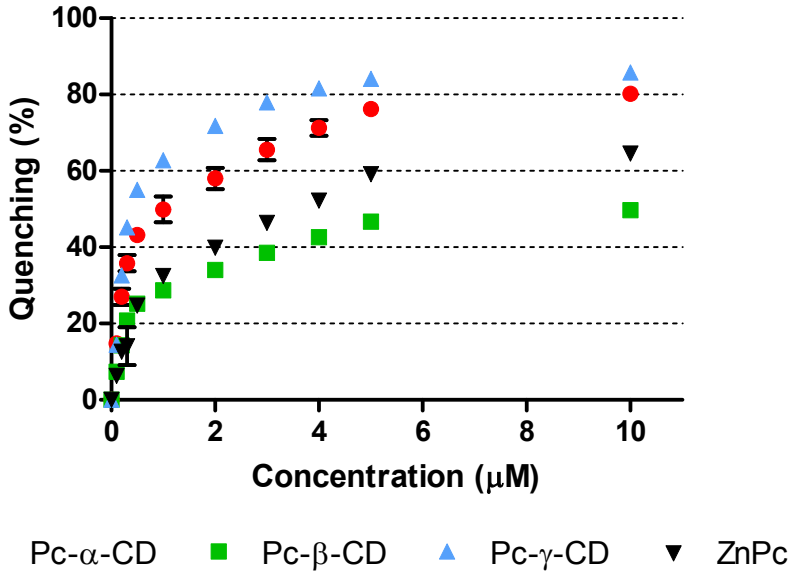


Figure SI 7– Emission quenching curves of 2 μM of HSA, after addition of the Pc-CDs **1-3** at concentrations between 0 to 10 μM. Quenching (%) = $(F_0\text{-}F)/(F_0)\times100$, where F_0 and F are the HSA fluorescence intensities in the absence and presence of the Pc-CDs ($\lambda_{exc.}$ at 280 nm and $\lambda_{emission}$ at 335 nm).

HSA demonstrates a characteristic emission maximum band at 335 nm (after excitation at 280 nm, Figure SI 8). The effects of DMSO on HSA fluorescence quenching were tested, since the stock solutions of the Pc-CDs were prepared in this organic solvent. Over a concentration range of 0-1% (v/v), DMSO did not quench HSA fluorescence (data not shown).

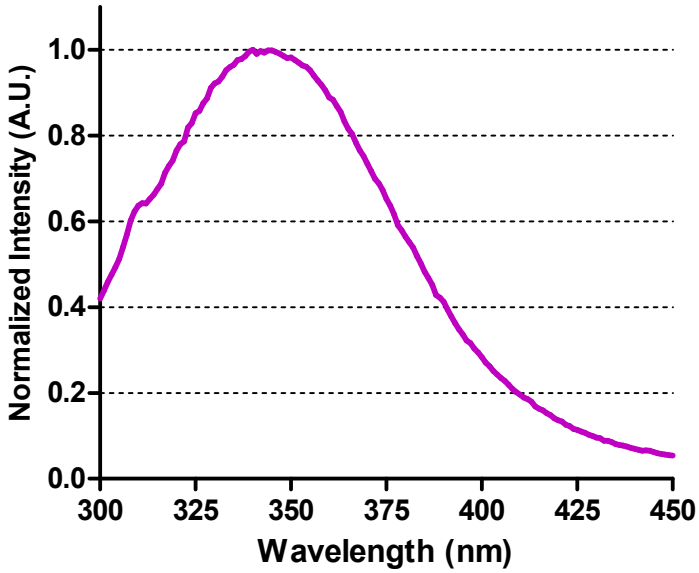


Figure SI 8 –Fluorescence emission spectra of HSA at 2 μM in PBS ($\lambda_{exc.}$ at 280 nm).

The emission and absorption spectra of Pc-CDs were also recorded after addition of HSA at 2 μM, keeping always the final concentration of DMSO below 1% (v/v). The emission spectra of Pc-CDs were acquired for the wavelength range of 650-850 nm upon excitation at 610 nm. The excitation and emission slits width were set at 2.0 nm.

Considering that Pc-CDs in PBS have an absorption band (Figure SI 1) in the region of the emission spectra of the HSA protein (Figure SI 8), we performed two additional experiments to demonstrate that HSA emission quenching was due to the interaction of HSA with Pc-CDs. On the first experiment, the emission spectra of Pc-CD solutions in the presence and absence of HSA were recorded after excitation at 610 nm (Figure SI 9). HSA by itself did not produce a signal over the spectral region (data not shown). Note the large increase in emission spectra associated with combining the HSA and the photosensitizer. The increase of Pc-CD emission suggests the formation of a complex that substantially enhances the emission characteristics of the photosensitizer. On the second experiment, the absorption spectra of Pc-CDs were recorded upon addition of HSA protein (Figure SI 10). After addition of HSA, it was observed hypochromicity without any Q-band shift, which unequivocally confirms the existence of interaction between HSA and Pc-CDs.

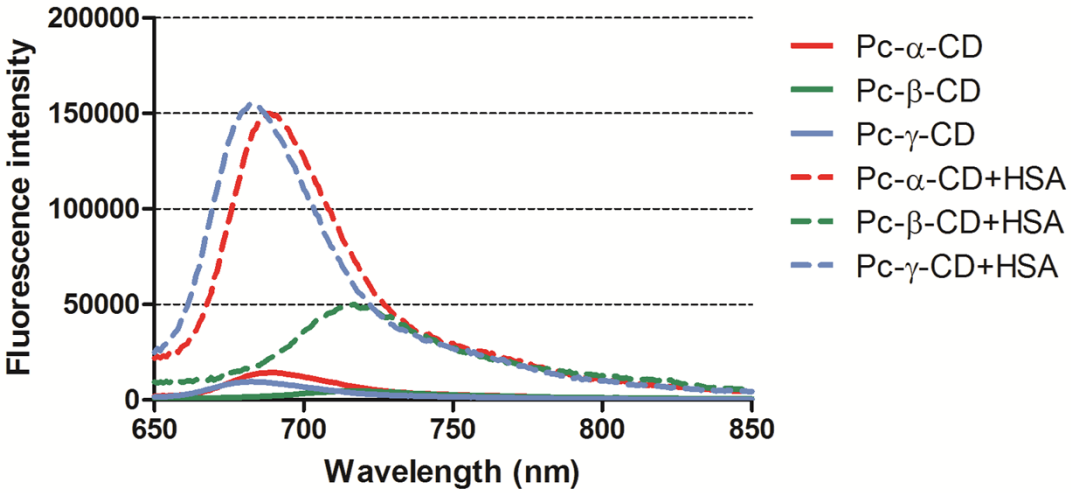


Figure SI 9 – Emission spectra of Pc-CDs in PBS before and after addition of HSA ($\lambda_{exc.}$ at 610 nm). The absorbance of Pc-CDs was adjusted to 0.5 (at 610 nm) before measurements.

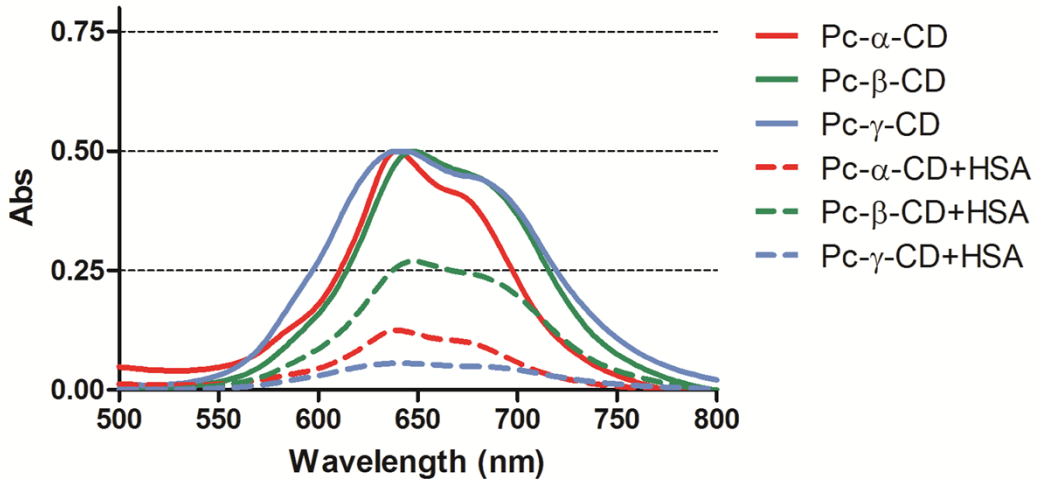


Figure SI 10 – Electronic absorption spectra of Pc-CDs in PBS before and after addition of HSA at 2 μM. The absorbance of Pc-CDs was adjusted to 0.5 (at maximum absorption wavelength) before measurements.

1.9 *In vitro* studies

1.9.1 Cell culture

Human bladder transitional cell carcinoma cell line UM-UC-3, established from urinary bladder of a male with a grade 3 bladder carcinoma (World Health Organization grading system classification), was

obtained from the American Type Culture Collection (ATCC®, Manassas, VA, USA). UM-UC-3 cells were grown in EMEM medium (ATCC) with Earle's Balanced Salt Solution, nonessential amino acids, sodium pyruvate, 10% (v/v) of heat-inactivated Fetal Bovine Serum (FBS; Life Technologies, Carlsbad, CA, USA) and antibiotic/antimicotic containing 100 units.mL^{-1} penicillin, 100 μg.mL^{-1} streptomycin and 0.25 μg.mL^{-1} amphotericin B (Sigma).

1.9.2 Determination of intracellular Pc-CD fluorescence by fluorescence microscopy

The UM-UC-3 bladder cancer cells were plated in coverslips at a density of 0.9 × 10^5 cells per mL for 24 h before treatment. Cells were incubated with 1 μM Pc-CD in PBS buffer (10 mM NaH_2PO_4, 70 mM Na_2HPO_4 and 145 mMNaCl at pH 7.6) for 3 h in the dark. Immediately after incubation, cells were, washed and, fixed with 4% paraformaldehyde (PFA) (Merck, Darmstadt, Germany) for 10 min at room temperature. The samples were rinsed in PBS, mounted using VectaSHIELD (Vector Laboratories, CA, Burlingame) mounting medium with DAPI, sealed around the perimeter with nail polish and stored at 4 ºC until visualization under the confocal microscope (LSM 710, Carl Zeiss).

1.9.3 Dark toxicity of Pc-CDs

Cells were seeded (9.4 × 10^4 cells.cm^{-2}) in 96-well cell culture plates and maintained in culture medium under an atmosphere of air containing 5% CO_2. After seeding the cells overnight, they were washed twice with PBS and incubated in darkness (at 37 ºC under an atmosphere of air containing 5% CO_2) with solutions of Pc-CDs in PBS. The cells were then washed twice with PBS and covered with 100 μL of fresh medium. After uptake, cells were incubated in a humidified incubator in an atmosphere containing 5% CO_2 and 95% air. After 24 h, cell toxicity was determined by the MTT colorimetric assay, using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT, yellow, Sigma). In this assay, the yellow colored MTT is reduced by mitochondrial dehydrogenases in living cells to a blue-colored formazan precipitate. The absorption of dissolved formazan correlates with the number of living cells.

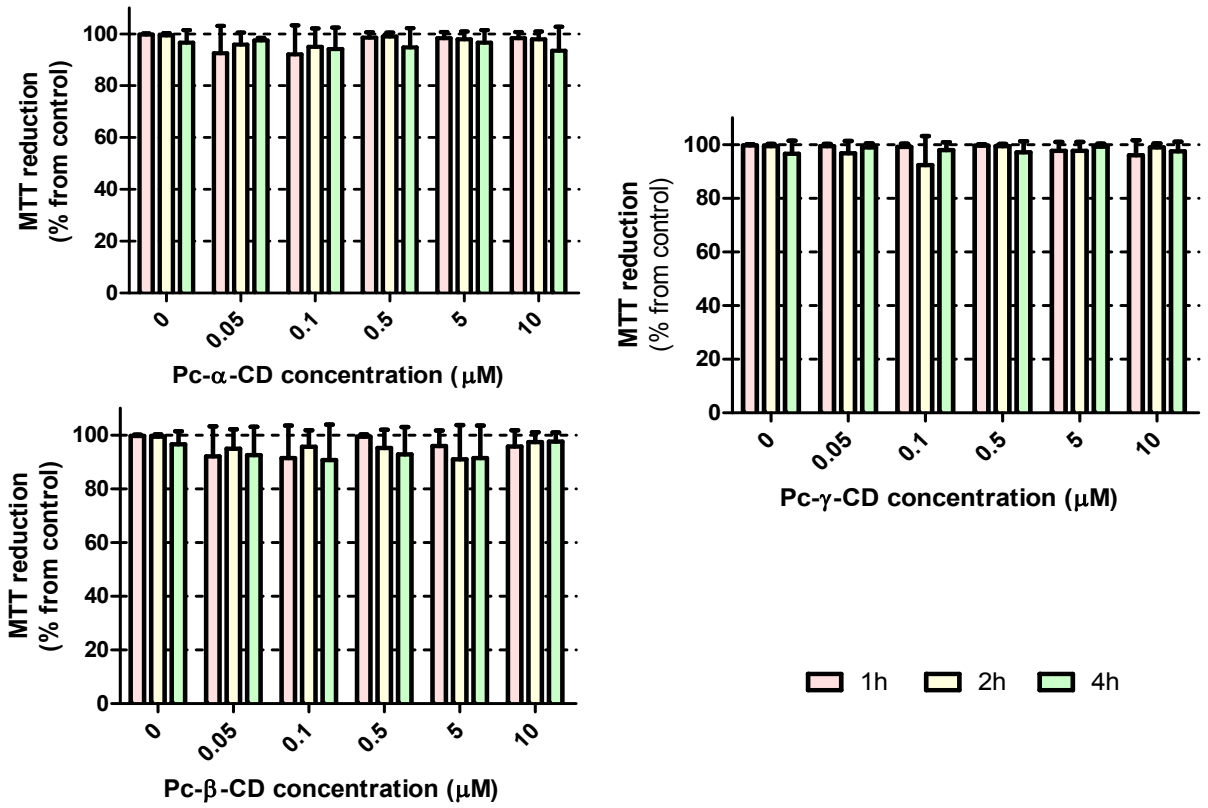


Figure SI 11 – Dark toxicity of Pc-CDs **1-3** in UM-UC-3 bladder cancer cells. Cells were incubated in darkness with Pc-CDs at different concentrations (0, 0.05, 0.1, 0.5, 5, and 10 μM in PBS buffer) for increasing uptake times (1, 2, and 4 h). Cytotoxicity was assessed 24 h after treatment using the MTT colorimetric assay. The percentage of cytotoxicity was calculated relatively to control cells (cells incubated with PBS in darkness) at the respective uptake time. Data are the mean value ± S.D. of at least three independent experiments performed in triplicates.

1.9.4 Phototoxicity of Pc-CDs

Cells were seeded (9.4×10^4 cells.cm^{-2}) in 96-well cell culture plates and maintained in culture medium under an atmosphere of air containing 5% of CO_2. After seeding the cells overnight, they were washed twice with PBS and incubated in darkness (at 37 ºC under an atmosphere of air containing 5% of CO_2) with solutions of Pc-CDs in PBS. The cells were then washed twice with PBS and covered with 100 μL of fresh medium. Cells were irradiated (using the illumination system referred above for the photostability assays) for 20 or 40 min with white (400-800 nm) and red light (620-750 nm) at a fluence rate of 50 mW.cm^{-2}. After irradiation, cells were incubated in a humidified incubator in an atmosphere containing 5% of CO_2 and 95% of air. After 24h of PDT, cell phototoxicity was determined

by the MTT colorimetric assay, using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT, yellow, Sigma).

The IC_{50} values (i.e. concentration of PS required to reduce cell viability by 50% as compared to the control cells) were calculated using non-linear regression analysis the sigmoidal dose-response curves (using GraphPad Prism).

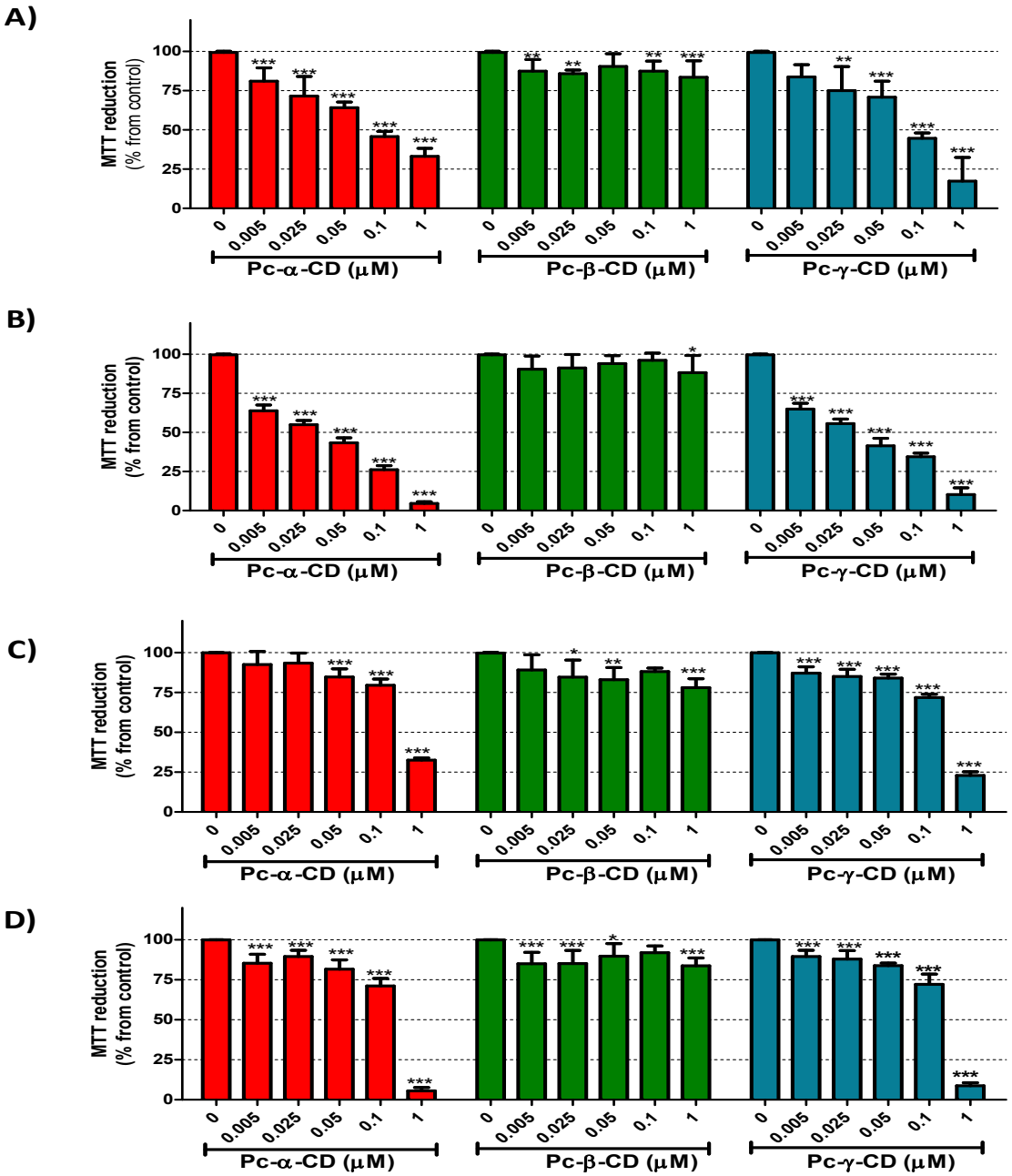


Figure SI 12 –Photocytotoxic effects after PDT with Pc-CDs **1-3** in UM-UC-3 bladder cancer cells. Cells were incubated in darkness with Pc-CDs **1-3** at different concentrations (0, 0.005, 0.025, 0.1 and

1 μM in PBS) for 3 h and irradiated with red light (620-750 nm) for 20 min (A) and 40 min (B), or with white light (400–800 nm) for 20 min (C) and 40 min (D) at a potency of 50 $mW.cm^{-2}$. Cytotoxicity was assessed 24 h after PDT using the MTT colorimetric assay. The percentage of phototoxicity was calculated relatively to control cells (cells incubated in darkness with PBS and then irradiated). Data are the mean value ± S.D. of at least three independent experiments performed in triplicates. *($p<0.05$), **($p<0.001$), ***($p<0.0001$) significantly different from control cells.

1.9.5 Reactive Oxygen Species (ROS) determination after PDT

Immediately after photodynamic treatment, cells were washed and incubated with 5 μM of H_2DCFDA (2′,7′-dichlorodihydrofluorescein) from Invitrogen (Carlsbad, CA, USA) in PBS, for 1 h at 37 °C and protected from light. This probe quantitatively reacts with several ROS to yield the fluorescent product DCF. The cells were mechanically scrapped in 130 μL of 1% (m/v) sodium dodecyl sulfate (SDS from Sigma) solution in PBS (pH 7.0) and the plate was stirred on an automatic plate shaker in the dark at room temperature. Cell suspension (100 μL) was transferred into a 96-well black plate (Greiner Bio-One) and used for fluorescence measurements of DCF. The measurements were performed using a microtiter plate reader (Synergy HT, Biotek Instruments) with the excitation and emission filters set at 485/20 nm and 528/20 nm, respectively. It was used 25 μL of the cell suspension to determine the protein concentration using the Pierce® BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

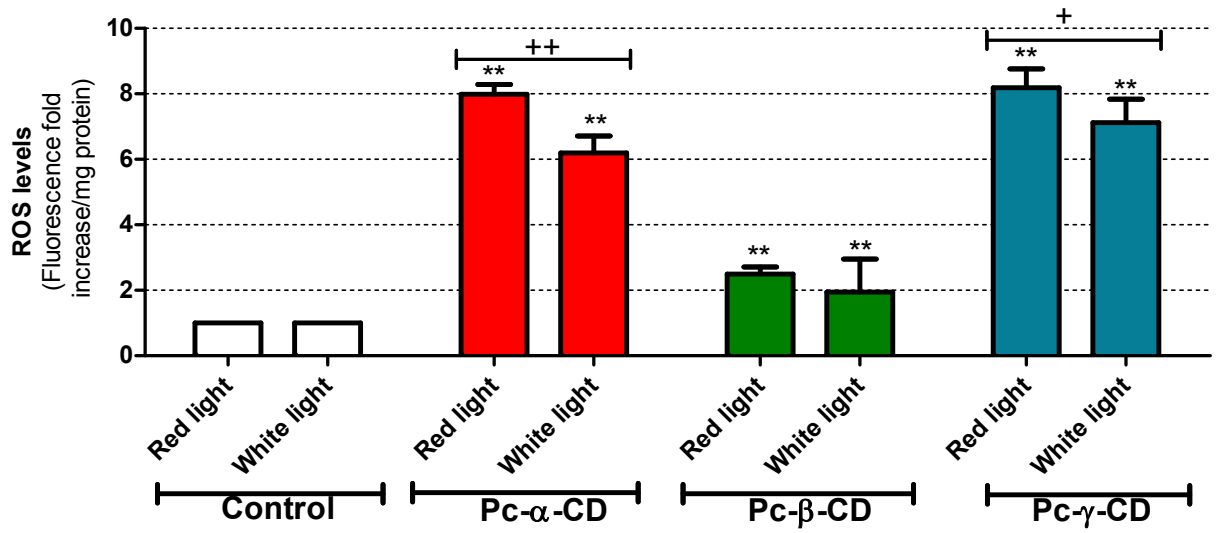


Figure SI 13 – Quantification of DCF fluorescence increase (as a measure of ROS production) after PDT. UM-UC-3 bladder cancer cells were incubated with 1 μM of Pc-CD for 3 h in the dark. After PDT with red (620-750 nm) or white light (400-800 nm) during 40 min, it was detected an increase in

intracellular oxidative stress observed by oxidation of the fluorescent probe H_2DCFDA (5 μM in PBS). **Significantly different from irradiated-control cells ($p<0.001$), $^{++}$significantly different from cells incubated with **Pc-α-CD** and irradiated with red light ($p<0.001$), $^{+}$significantly different from cells incubated with **Pc-γ-CD** and irradiated with red light ($p<0.05$).

1.9.6 Statistical analysis

GraphPad Prism (v.5.00, GraphPad Software) was used to perform the statistical analysis. The Mann-Whitney non-parametric test was applied to determine the differences between the selected two groups. Level of significance was set at $p < 0.05$.

1.10 ^{19}FNMR spectra of Pc-CD conjugates

The low resolution observed in the ^{1}H and ^{19}F NMR spectra and the overlap of signals, even at 60 ºC, did not allow the adequate assignment of the protons and fluorine atoms, respectively. However, the ^{19}F NMR spectra of Pc-CDs **1**-**3** in DMSO-d_6 show the presence of two multiplets around δ -167 to -164 and -179 to -176 ppm, corresponding respectively to the six and eight β- and α-fluorine atoms of the Pc moiety (Figs. SI 14-16).

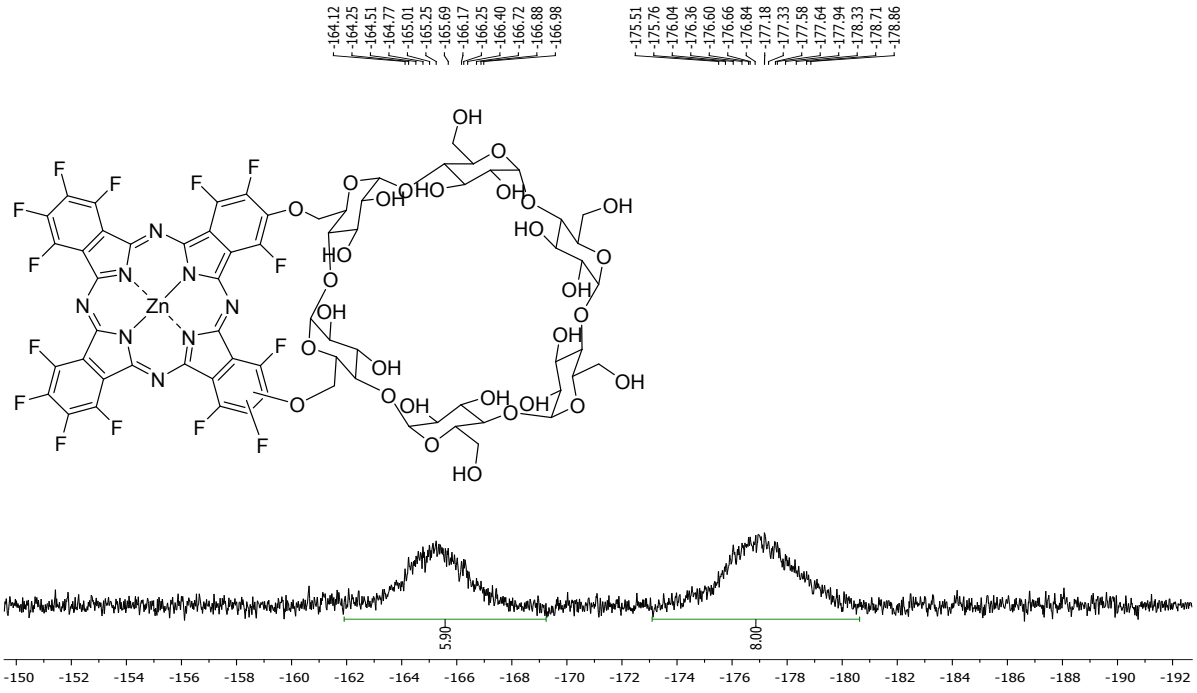


Figure SI 14–^{19}F NMR spectroscopy of **Pc-α-CD1**.

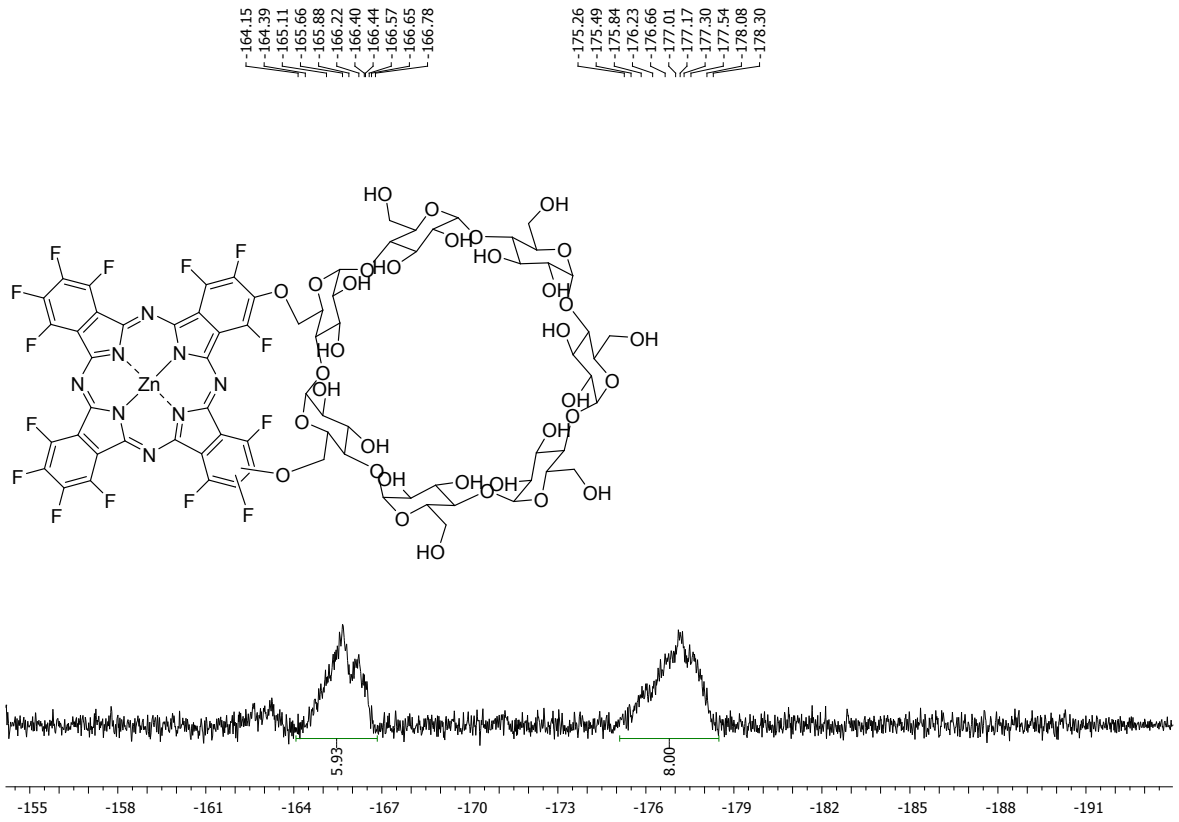


Figure SI 15–^{19}F NMR spectroscopy of **Pc-β-CD2**.

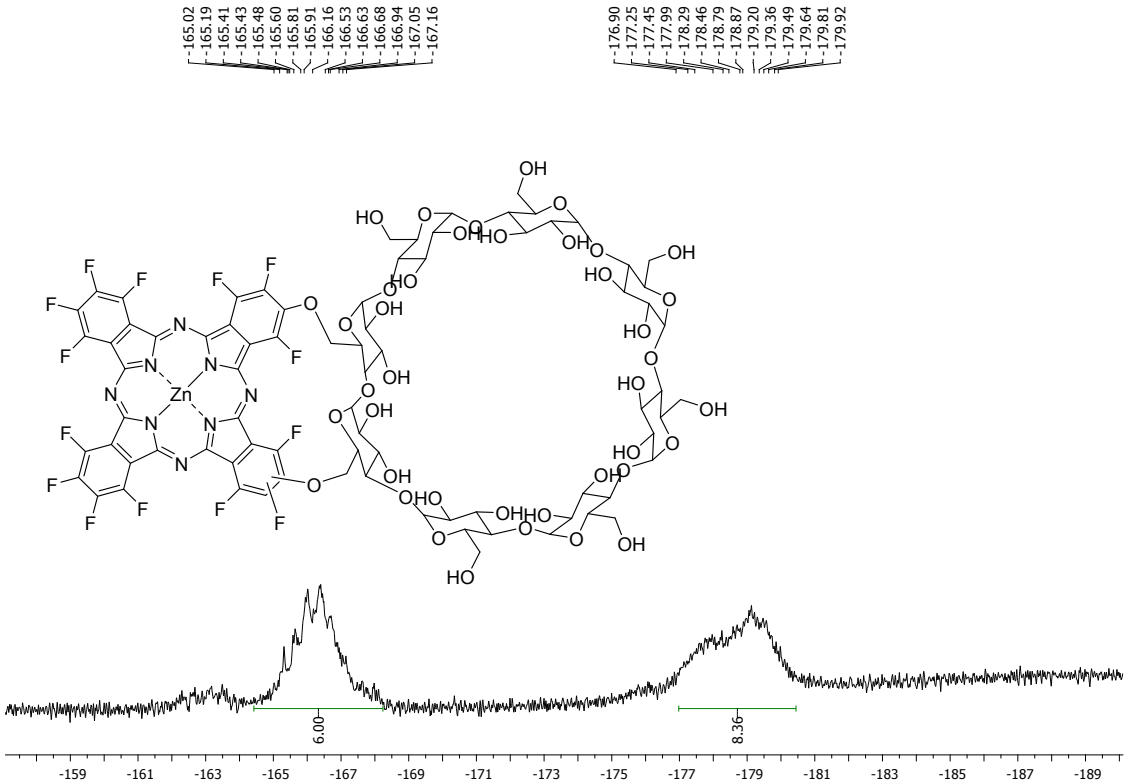


Figure SI 16–^{19}F NMR spectroscopy of **Pc-γ-CD3**.

1.11 MALDI spectra of Pc-CD conjugates

MALDI-MS and MALDI-MS/MS spectra were acquired to confirm the structural assignment of each $[M+Na]^+$ ion of **Pc-α-CD**, **Pc-β-CD** and **Pc-γ-CD**, respectively, and the spectra are displayed in Figures 2-4. The MS/MS spectra of Pcs **1-3** showed a characteristic fragmentation owing to the preferential cleavage, whose the sequential loss number depends on the CD type coupled to the β-position of Pcs macrocycle.

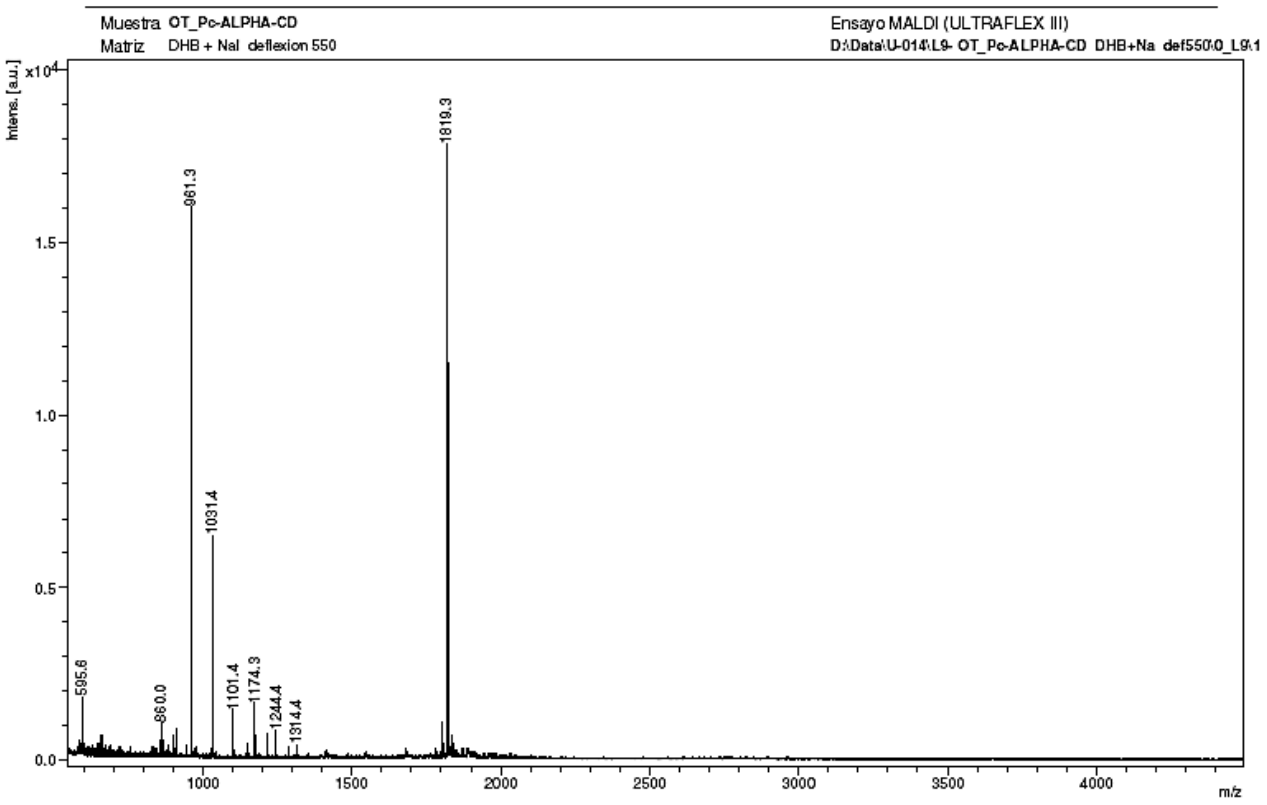


Figure SI 17 – MALDI-MS spectrum of the ion $[M+Na]^+$ at *m/z* 1819.3 of **1** (**Pc-α-CD**).

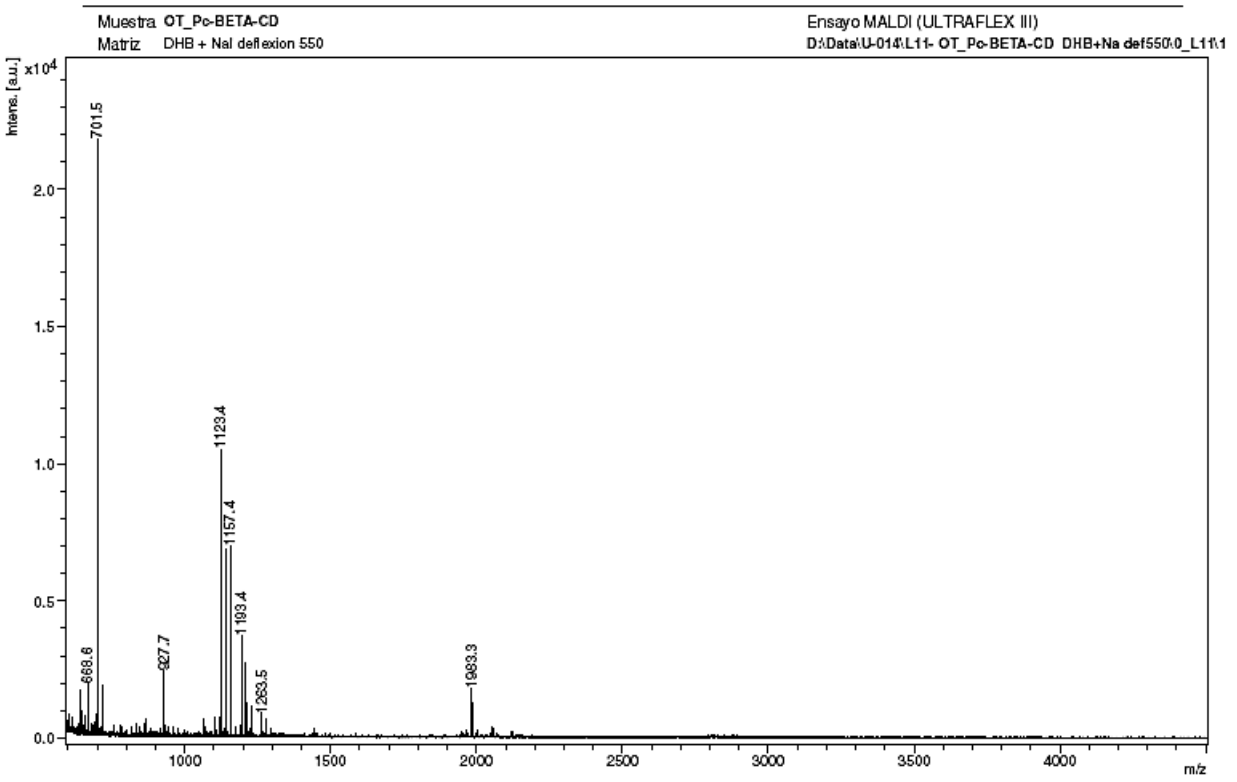


Figure SI 18 – MALDI-MS spectrum of the ion $[M+Na]^+$ at *m/z* 1983.3 of **2** (**Pc-β-CD**).

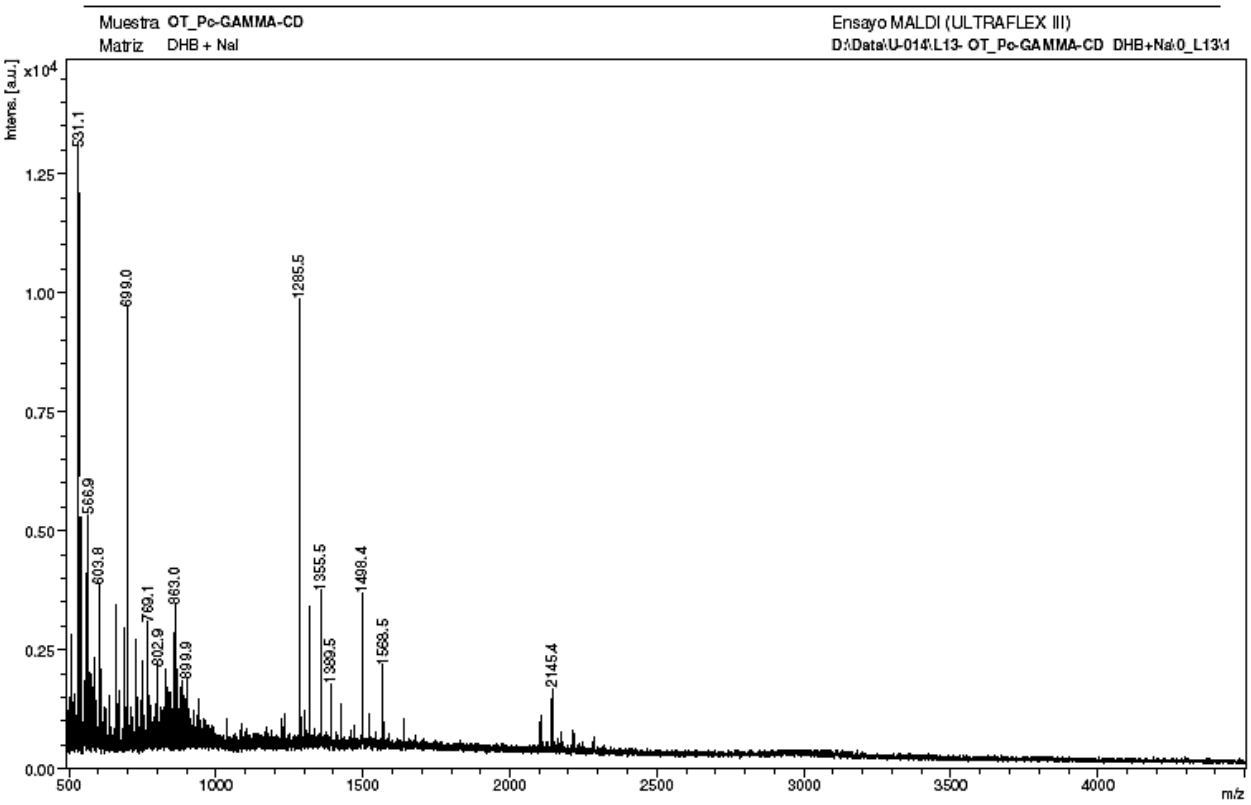


Figure SI 19 – MALDI-MS spectrum of the ion $[M+Na]^+$ at *m/z* 2145.4 of **3** (**Pc-γ-CD**).

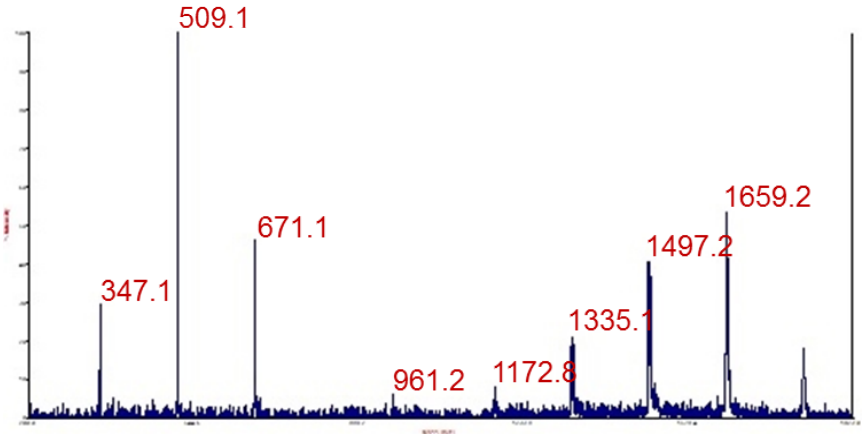


Figure SI 20 – MALDI-MS/MS spectrum of the ion $[M+Na]^+$ at *m/z* 1821.2 of **1** (**Pc-α-CD**).

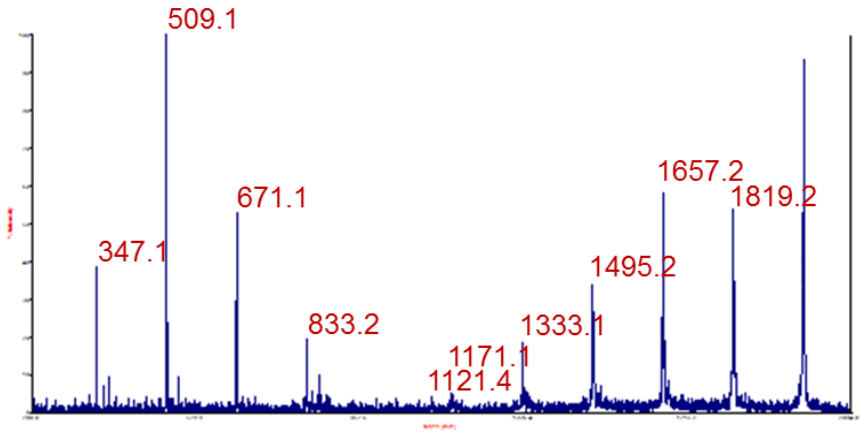


Figure SI 21 – MALDI-MS/MS spectrum of the ion $[M+Na]^+$ at *m/z* 1981.2 of **2** (**Pc-β-CD**).

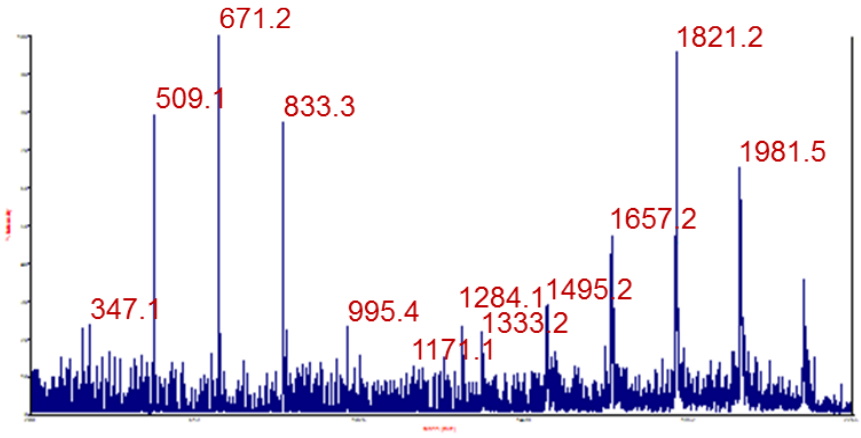
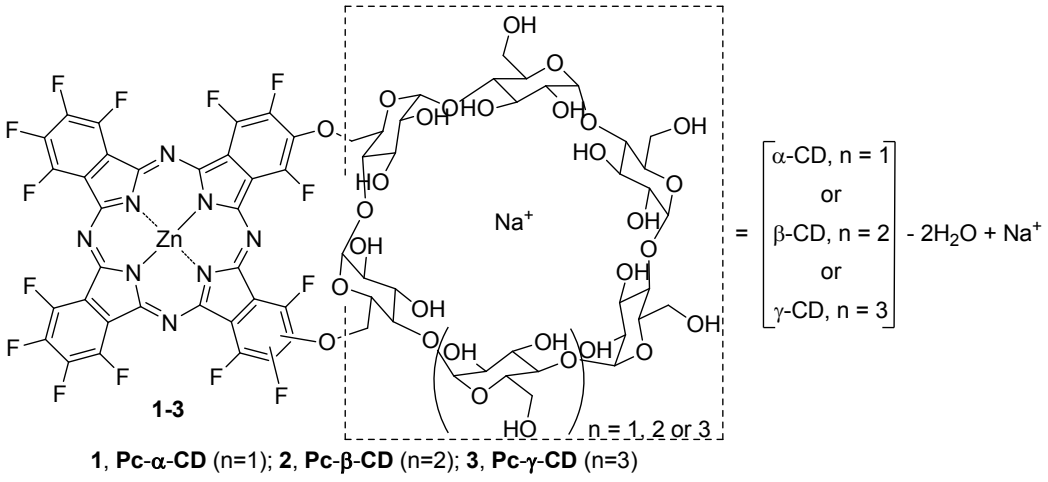


Figure SI 22 – MALDI-MS/MS spectrum of the ion $[M+Na]^+$ at *m/z* 2143.3 of **3** (**Pc-γ-CD**).

The results obtained in the MALDI MS/MS spectra of Pc-CDs **1-3** are summarized in Table 1. All MS/MS spectra demonstrated losses of one to *n*-hexose residues (-162 Da, loss of $C_5H_{10}O_5 \approx Hex_{res}$) with maximum sequential losses of 4, 5 and 6 hexose units for [**Pc-α-CD**+Na-4Hex_{res}]$^+$ (*m/z* 1173), [**Pc-β-CD**+Na-5Hex_{res}]$^+$ (*m/z* 1171) and [**Pc-γ-CD**+Na-6Hex_{res}]$^+$ (*m/z* 1171), respectively. This pattern of fragmentation confirms the presence of the CD moiety linked to the Pc. These fragmentations are rather interesting, since the maximum number of lost hexoses is equal to the number of hexose residues present in the CD (6, 7 and 8 Hex_{res} for **α-**, **β-** and **γ-CDs**, respectively) minus 2. This information confirms a scission of the structure for the Pc-CD derivatives since the two sugar units linked to the Pc core are more difficult to be eliminated. Other fragmentation pathways owing to the cleavage between the Pc and CD units lead to the formation of the product ions correspondent to the CD moiety at *m/z* 961, 1121 and 1284 for **Pc-α-CD**, **Pc-β-CD** and **Pc-γ-CD**, respectively, but with minus two water molecules ([CD-2H_2O+Na]$^+$) which confirms the size of the CD linked to Pc (Figure 5). The formation of these product ions rather the total CD is due to the favored cleavage in the sugar units between the C_6 and the O that occurs in both sides. This leads to a product ion, with a mass equal to the mass of the sugar minus 18 (sugar-H_2O). Since this type of cleavages occur twice in these Pc-CDs, the product ion obtained in the MS/MS spectra was identified as [CD-2H_2O+Na]$^+$.In addition, the formation of sodium adducts of oligosaccharides [Hex_{res}*n*+Na]$^+$ (*n* = 2-4, 5 or 6 for **Pc-α-CD**, **Pc-β-CD** and **Pc-γ-CD**, respectively) also confirms the structural identification of these Pc derivatives. This fragmentation pattern occur at every acetal connection of the opened CD concerning to *n* (*n* = 1-6) monomeric units respectively.



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

Figure SI 20 – Representation of the cleavage between Pc and CD for Pc-CD dyads **1-3**.

Table SI 2 – Results of MS/MS experiments performed for **1** (**Pc-α-CD**), **2** (**Pc-β-CD**) and **3** (**Pc-γ-CD**) ionized by MALDI-TOF-MS.

Produced ions form	Neutral loss (Da)	1, Pc-α-CD *m/z* (%RA$^{Q\text{-}TOF}$)	2, Pc-β-CD *m/z* (%RA$^{Q\text{-}TOF}$)	3, Pc-γ-CD *m/z* (%RA$^{Q\text{-}TOF}$)
[PcCD+Na]$^+$		1821	1981	2143
[PcCD+Na-1Hex$_{res}$]$^+$	-162	1659 (55)	1819 (55)	1981 (65)
[PcCD+Na-2Hex$_{res}$]$^+$	-324	1497 (40)	1657 (60)	1821 (100)
[PcCD+Na-3Hex$_{res}$]$^+$	-486	1335 (20)	1495 (35)	1657 (48)
[PcCD+Na-4Hex$_{res}$]$^+$	-648	1173 (10)	1333 (20)	1495 (30)
[PcCD+Na-5Hex$_{res}$]$^+$	-810	-----	1171 (7)	1333 (20)
[PcCD+Na-6Hex$_{res}$]$^+$	-972	-----	-----	1171 (12)
[CD+Na-2H$_2$O]$^+$	-860	961 (5)	1121 (5)	1284 (22)
[Hex$_{res6}$+Na]$^+$	-1148	-----	-----	995 (22)
[Hex$_{res5}$+Na]$^+$	----- /-1148/-1310	-----	833 (20)	833 (78)
[Hex$_{res4}$+Na]$^+$	-1150/-1310/-1472	671 (45)	671 (55)	671 (100)
[Hex$_{res3}$+Na]$^+$	-1312/-1472/-1634	509 (100)	509 (100)	509 (80)
[Hex$_{res2}$+Na]$^+$	-1474/-1634/-1796	347 (30)	347 (38)	347 (25)

In summary, MS/MS MALDI–TOF mass spectrometry proved to be an excellent tool to distinguish the Pc-CD dyads **1-3**, requiring very low quantities of sample without the need of sample manipulation. The MS/MS spectra of these samples allow differentiation of gradual loss of sugar units, and confirm the structure of all three derivatives.