

Photophysical properties of a photocytotoxic fluorinated chlorin conjugated to four β -cyclodextrins†

João Nuno Silva,^a Ana M. G. Silva,^{‡,b} João P. Tomé,^b Anderson O. Ribeiro,^c M. Rosário M. Domingues,^b José A. S. Cavaleiro,^b Artur M. S. Silva,^b M. Graça P. M. S. Neves,^b Augusto C. Tomé,^b Osvaldo A. Serra,^c Francisco Bosca,^d Paulo Filipe,^a René Santus^e and Patrice Morlière*^{f,g}

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A *meso*-tetrakis(pentafluorophenyl)-chlorin with the reduced pyrrole ring linked to an isoxazolidine ring (FC) has been conjugated to four β -cyclodextrins (CDFC). The CDFC exhibits excellent water solubility and is a potent photosensitizer towards proliferating NCTC 2544 human keratinocytes. The study by conventional steady state absorption and fluorescence spectroscopies and by time-resolved femto- and nanosecond laser flash spectroscopies suggests that in ethanol and pH 7 buffer the β -cyclodextrins embed the highly hydrophobic tetrakis(pentafluorophenyl)-chlorin macrocycle and strongly interact with the chlorin rings in the singlet and triplet manifolds. In these solvents, femtosecond spectroscopy suggests that the conjugate undergoes a rapid relaxation in the upper excited singlet states induced by photochemical and/or conformation change(s) at a rate of about 5 ps⁻¹ to fluorescent states whose lifetime is \sim 8 ns. This interaction is destroyed upon addition of Triton X100 to buffer. Both FC and CDFC strongly fluoresce ($\Phi_F \sim 0.5$) in micelles. Similar behavior is observed at the triplet level. In ethanol and water, the initial transient triplet state absorbance decays within 1–3 μ s yielding a longer lived triplet with spectral properties indistinguishable from that of original difference absorbance spectra. The determination of the molar absorbance in the 440–460 nm region (\sim 35 000 M⁻¹ cm⁻¹) leads to an estimate of \sim 0.2 for the triplet formation quantum yield of FC in toluene and of FC and CDFC in Triton X100 micelles. Quenching of the CDFC triplets by dioxygen in buffer produces ¹O₂ in a good yield consistent with the effective photocytotoxicity of the chlorin–cyclodextrins conjugate towards cultured NCTC 2544 human keratinocytes. By contrast, FC which aggregates in buffer produces little if any ¹O₂.

Introduction

Photodynamic therapy (PDT) is an alternative treatment to pre-existing therapies in several domains of oncology and ophthalmology. Presently authorized photoactive substances are tetrapyrrole

derivatives producing singlet oxygen as the major cytotoxin upon illumination of the tumor site with visible light after systemic administration of a photosensitizer.^{1–3} In dermatology, the treatment of basal and squamous cell carcinoma and of the pre-cancerous lesions of solar keratoses by PDT involves topical application of the esters of δ -aminolevulinic acid, the precursor of protoporphyrin IX.⁴ Most effective tetrapyrroles are lipophilic and are carried by blood lipoproteins which deliver them not only to the tumor but also to every organ.^{5,6} The poor water solubility of tetrapyrroles is another limiting factor implying the preparation of special formulations for their systemic or topical administration. Several strategies are currently used to overcome these difficulties. The synthesis of porphyrins and chlorins bearing water soluble groups conferring tumor selectivity, such as tumor-specific antibodies or peptides and sugars has been carried out.^{7,8} Accordingly, the synthesis of β -cyclodextrin conjugates of porphyrins for dermatological applications is of interest since cyclodextrins have been used to improve local and systemic dermal drug delivery by enhancing drug release and/or permeation and the reduction of drug degradation in topical preparations. In addition, cyclodextrins reduce drug-induced local irritation.^{9–13}

It has been previously shown that a bis- β -cyclodextrin derivative of *meso*-tetrakis(pentafluorophenyl)-porphyrin which specifically binds to serum albumin was the most effective PDT

^aUniversidade de Lisboa, Faculdade de Medicina, Clínica Universitária de Dermatologia, 1699, Lisboa, Portugal

^bUniversity of Aveiro, Department of Chemistry, Campus Universitário de Santiago, 3810-193, Aveiro, Portugal

^cUniversity of São Paulo, Department of Chemistry, FFCLRP, Ribeirão Preto, Brazil

^dUniversidad Politécnica de Valencia, Instituto de Tecnología Química, 46022, Valencia, Spain

^eINSERM, U 697, Institut de Recherche sur la Peau, 75475 Paris, France; Muséum National d'Histoire Naturelle, Département RDDM, 75231, Paris, France

^fINSERM ERI12, 80054 Amiens, France; Université de Picardie Jules Verne, Faculté de Médecine et de Pharmacie, 80036, France; CHU Amiens, Laboratoire de Biochimie, 80054 Amiens, France

^gMailing address: CHU Amiens Nord, Laboratoire de Biochimie, INSERM ERI12, place Victor Pauchet, 80054 Amiens Cedex 1, France. E-mail: morliere.patrice@chu-amiens.fr; Fax: +33 3 22 66 89 17; Tel: +33 3 22 66 86 69

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‡ University of Porto, Faculty of Science, REQUIMTE, Department of Chemistry, 4169-007 Porto, Portugal.

agent when administered to mice bearing aggressive subcutaneous tumors induced by implanted mammary carcinoma cells.¹⁴ While the conjugation with cyclodextrin provides fair water solubility, fluorination of porphyrinoid photosensitizers enhances their photodynamic activity.¹⁵ It is therefore interesting to study the contribution of cyclodextrin conjugation and of fluorination on the physico-chemical basis of this increased photosensitizing potency. Along these lines, we have synthesized a *meso*-tetrakis(pentafluorophenyl)-chlorin bearing a *N*-benzylisoazolidine ring (FC) conjugated to four β -cyclodextrins (CDFC) to further assess the potential interest of this new class of chlorins as PDT photosensitizers. In the light of the effective photocytotoxic effectiveness of CDFC towards cultured human keratinocytes and to help understanding the photobiological properties of CDFC we have also undertaken a study of the photophysics of these fluorinated tetrapyrrole derivatives. We have determined their excited singlet and triplet state properties by means of time-resolved femto and nanosecond spectroscopies. A micro-environmental-dependent interaction of the cyclodextrin moieties with the fluorinated chlorin ring in the singlet and triplet excited states is observed in buffer and ethanol but it is abolished upon incorporation of CDFC into neutral Triton X100 micelles. In buffered aqueous solutions, the presence of four β -cyclodextrin macrocycles prevents CDFC from stacking interactions between hydrophobic chlorin macrocycles leading to a long-lived triplet state which forms $^1\text{O}_2$ in a good yield upon quenching by oxygen.

Materials and methods

Chemicals

Reagents for cell culture, minimum essential medium with Earle's salts (EMEM), Hanks' buffered saline solution containing 20 mM Hepes (HBSS), Dulbecco's phosphate-buffered saline (PBS), all without phenol red, fetal calf serum (FCS), trypsin and antibiotics were purchased from Gibco (Cergy-Pontoise, France). L-Histidine (His) and the Folin reagent were supplied by Sigma Chemical Co. (St Louis, MO, USA). Neutral Red (NR) was a Fluka (Saint-Quentin Fallavier, France) product whereas Triton X100 (TX100), sodium dodecyl sulfate (SDS), absolute ethanol and DMSO (spectroscopic grade solvents) were supplied by Merck (Darmstadt, Germany). All other chemicals and solvents used in this work were of the purest available grade and were used without further purification. The phosphate buffer (10 mM, pH 7) was prepared in pure water obtained with a reverse osmosis system from Ser-A-Pure Co. The water exhibits a resistivity of $>18 \text{ M}\Omega \text{ cm}^{-1}$ and a total organic content of $<10 \text{ ppb}$.

Synthesis of the chlorins FC and CDFC

Synthesis of chlorin FC. The 1,3-dipolar cycloaddition reaction of *meso*-tetrakis(pentafluorophenyl)-porphyrin, (prepared by a published procedure)¹⁶ with *N*-benzylnitron, generated *in situ* from paraformaldehyde and *N*-benzylhydroxylamine hydrochloride, in the presence of K_2CO_3 , led to chlorin FC (Fig. 1). Briefly, a toluene solution (2 mL) of *meso*-tetrakis(pentafluorophenyl)-porphyrin (28.2 mg, 0.03 mmol), *N*-benzylhydroxylamine hydrochloride (18.4 mg, 0.11 mmol), paraformaldehyde (8.7 mg, 0.29 mmol) and K_2CO_3 (32.0 mg, 0.23 mmol) was heated at 60°C ,

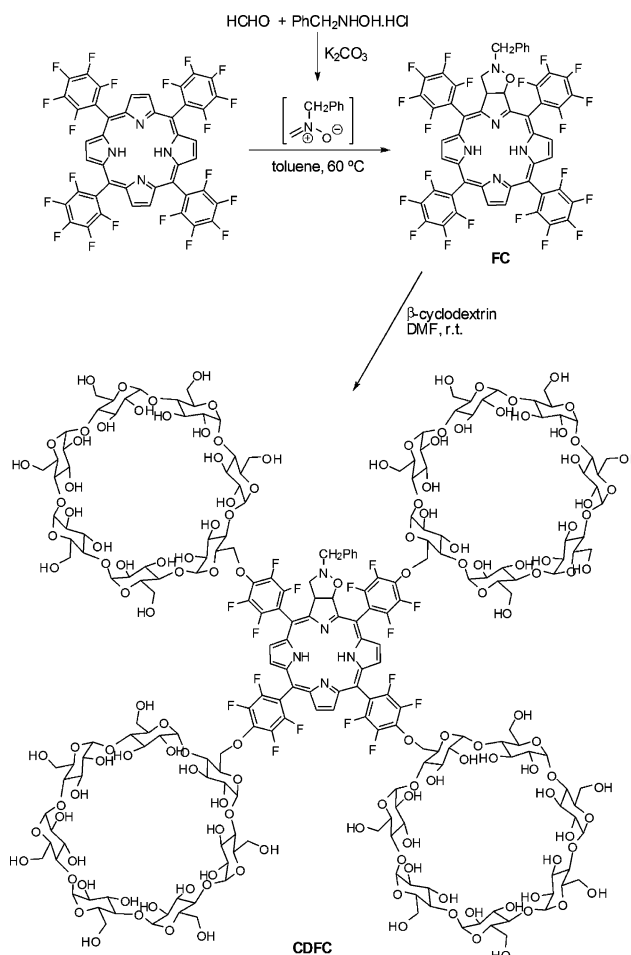


Fig. 1 Reaction scheme and structure of FC and CDFC.

under a nitrogen atmosphere, for 6 d. After further addition of *N*-benzylhydroxylamine hydrochloride (18.4 mg), paraformaldehyde (8.7 mg) and K_2CO_3 (32.0 mg), the resulting mixture was heated for two more days. The reaction mixture was cooled, filtered and washed with CH_2Cl_2 . The solvents were evaporated to dryness and the residue was purified by flash chromatography using hexane- CH_2Cl_2 (2:3) as the eluent. The unchanged starting porphyrin (4.2 mg, 15%) was the first fraction to be collected, followed by the chlorin FC (16.5 mg, 52% yield).

$^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ : -2.08 (s, 2 H, NH), 3.20–3.88 (m, 4 H), 5.40–5.50 (m, 1 H), 6.80–7.19 (m, 6 H), 8.45–8.55 (m, 4 H), 8.77 (broad s, 2 H). ESI-MS m/z : 1110 $[\text{M} + \text{H}]^+$ (see electronic supplementary information, ESI ‡).

Synthesis of chlorin CDFC. A solution of chlorin FC (16.5 mg, 0.02 mmol) and β -cyclodextrin (200 mg, 0.17 mmol) in dry DMF (5 mL) was stirred at room temperature, under a nitrogen atmosphere, in the presence of K_2CO_3 (24.0 mg, 0.17 mmol), for 5 d. After that time, the reaction mixture was washed with an aqueous solution of citric acid and then neutralised with NaHCO_3 . The resulting mixture was purified by a reverse-phase chromatography using $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ (1:4) to elute the chlorin CDFC. The chlorin CDFC was crystallised from acetone to give 20.0 mg (24% of yield) of a dark green powder. MALDI-MS m/z : 5571 $[\text{M} + 2\text{H}]^+$ (see ESI ‡).

Laser flash spectroscopy

Femtosecond transient absorbance measurements were conducted at the Radiation Laboratory of the University of Notre Dame (Indiana, USA) using a Clark6MXR 2010 laser system transient and an optical detection system provided by Ultrafast Systems (Helios). The source for the pump and probe pulses is the fundamental of the Clark laser system (775 nm, 1 mJ pulse⁻¹, FWHM = 130 fs, 1 kHz repetition rate). A second harmonic generator, is introduced into the path of the laser beam to provide 387 nm (3.20 eV, 130 fs) laser pulses for the pump. 5% of the fundamental is used to create a white light continuum. Before creating the white light probe the fundamental is fed through a delay providing the experimental time window of 1.6 ns with a maximum step resolution of 7 fs. The energy of the pump beam (2 mm diameter) is 5 μ J pulse⁻¹ on the sample cell (2 mm light path) where it merged with the analyzing white light with an angle <10°. After passing through the cell the white light is focused on a 200 μ m core fiber connected to a CCD spectrograph enabling time-resolved spectra recording (425–800 nm). Usually, 5 000 excitation pulses are averaged to obtain the transient spectrum at a set delay time. Kinetics at appropriate wavelengths are obtained from the time resolved spectra. The 3D data are analyzed with the surface Explorer lite software.¹⁷

Nanosecond laser flash experiments were carried out using the 3rd harmonic ($\lambda_{\text{exc}} = 355$ nm) of a Quantel pulsed Nd:YAG spectrum laser system instrument. The single pulses were *ca.* 10 ns duration, and the energy was *ca.* 15 mJ pulse⁻¹ at the laser output. A Xenon lamp was employed as detecting light source. The laser flash photolysis apparatus consisted of the pulsed laser, the Xe lamp, a monochromator, and a photomultiplier (PMT) system made up of side-on PMT, PMT housing, and a PMT power supply. The output signal from the Tektronix oscilloscope was transferred to a personal computer for study. Samples were contained in 10 \times 10 mm cells made of Suprasil quartz and were deaerated for at least 20 min with dry nitrogen or, when desired, saturated with oxygen prior to the experiments. Decays were generally recorded after automatically averaging 6 to 8 traces whereas for transient spectrum acquisition absorbance (generally 5 nm intervals) resulted from averaging 3–4 data at each wavelength.

All laser spectroscopic measurements were conducted at room temperature with solutions of the photosensitizers whose absorbance was *ca.* 0.3 (387 nm) and 0.4 (355 nm).

Routine equipment

Flash chromatography was carried out using silica gel Merck (230–400 mesh), and the preparative thin layer chromatography was carried out with 20 \times 20 cm glass plates coated with silica gel 60 (1 mm thick). Analytical TLC was carried out with pre-coated sheets with silica gel (0.2 mm thick, Merck). Waters Sep-Pak[®] Vac 35 cc (10 g) cartridges were used for solid phase extraction.

UV/Vis absorption measurements were performed with either a UVIKON 943 or a Shimadzu UV-2101PC spectrometer. A SLM AMINCO-BOWMAN (series 2) (Bioritech, Chamarande, France) fluorometer equipped with excitation and emission correction spectra was used for fluorescence measurements. Fluorescence spectra and quantum yields were obtained with solutions whose absorbance at the excitation wavelength (usually 407 nm) was

<0.1. *meso*-Tetraphenylporphyrin was used as a reference for which $\Phi_{\text{F}} = 0.12$ in toluene.¹⁸ *meso*-Tetraphenylporphyrin is particularly suitable as a reference quantum counter for FC and CDFC since its absorbance and fluorescence spectra are close to those of the chlorins.

Fluorescence lifetimes were measured from optical cells with a light path of 5 mm containing solutions of photosensitizers whose absorbances were ~ 0.5 using the Horiba-Jobin Yvon NanoLed single photon counting system with excitation at 373 nm with 200 ps laser pulses. The emission wavelength was set at 654 nm. Fluorescence decay measurements were further analyzed using the IBH software library.

Photosensitized histidine degradation

Air-saturated 10 mM phosphate buffer solutions (pH 7) containing 500 μ M His and 5 μ M FC or CDFC or *meso*-tetrasulfonatophenyl porphyrin (TPPS₄) were irradiated with increasing light doses at 365 nm. Histidine was monitored by HPLC using a Whatman Partisil 10/25 SCX cation exchange column and 15 mM NH₄H₂PO₄ whose pH was adjusted to 2.3 by addition of phosphoric acid as mobile phase, as earlier described.¹⁹ Irradiations, in a 1 \times 1 cm cuvette (2.5 mL), were performed with an OSRAM HBO 200 W high pressure mercury lamp whose 365 nm Hg emission line was isolated as detailed before.¹⁹ Chemical actinometry based on the photoreduction of ferrioxalate by the UV radiation was carried out according to Parker.²⁰

Cell culture and treatment

The NCTC 2544 immortalized human skin keratinocyte cell line was purchased from ICN Flow (Fontenay sous Bois, France). Cultures were propagated in EMEM supplemented with 10% FCS, 100 U mL⁻¹ penicillin and 100 μ g mL⁻¹ streptomycin without other additives (weekly passages, 1 : 10 splitting ratio). Cells from trypsinized confluent monolayers were seeded at about 75 000 cells per 35 mm diameter plastic Petri dish containing 2.5 mL of EMEM supplemented with FCS. They were grown for 4 d at 60–80% of confluence. Cells were incubated for various times with 1 mL of medium (10% FCS-supplemented EMEM) containing the desired concentration of CDFC. After incubation, cells were thoroughly washed twice with 4 mL of HBSS before further use (photosensitizer uptake photocytotoxicity). Irradiations were carried out with 1 mL of HBSS, devoid of photosensitizer, covering the cell monolayers. Sham-irradiated cells, used as controls, consisted in cells kept in the dark for the same duration and under the same environmental conditions as the irradiated cells.

Cellular uptake of CDFC

Immediately after washing, cells were mechanically scraped in 1 mL of water. After collection, 100 μ L of an 11% SDS solution in 10 mM, pH 7.0 phosphate buffer were added to the cell suspension. Fifty μ L of this solution were saved for protein determination using the method of Lowry with the Folin reagent.²¹ The rest was used for fluorometric measurement of the photosensitizer concentration, using photosensitizer solutions of known concentrations for calibration with excitation and emission wavelengths respectively set at 409 and 649 nm. The photosensitizer concentration was

normalized to the protein content and the data are the mean \pm standard deviation (SD) of at least three independent experiments, each performed in triplicates.

Irradiation

Irradiation of cell monolayers with broad band red light was carried out with a custom-built table consisting of two 300 W tungsten-halogen lamps whose light was filtered with Balzers Y54 and callflex 3000 optical filters. The lamps were placed below the 30 \times 25 cm table thermostated at 37 $^{\circ}$ C. Under these conditions, most of the light arises from wavelengths in the range 500–750 nm as determined with a CS 1000A Minolta spectroradiometer. The methodology used for absolute calibration of the light fluence rate (15.2 mW cm $^{-2}$) has been previously published in detail.²²

Neutral Red uptake assay

As detailed earlier, the photocytotoxic effect was determined by the NR uptake assay.²² Challenged cells were washed and further incubated at 37 $^{\circ}$ C for 3 h with the supplemented culture medium before loading with NR. This 3 h lag was chosen to allow the initial damage to propagate but was short enough to avoid important proliferation of undamaged cells (population doubling time is about 1 day) which may alter data. The NR uptake is a widely used assay for evaluating the photocytotoxicity of exogenous drugs or hazardous compounds. It has been validated by the European Union for testing phototoxic chemicals and for the classification and labelling of hazardous chemical (EU Commission Directive 2000/33/EC).^{22,23} Data are presented as the percentage of NR uptake with respect to a control consisting of sham-irradiated untreated cells. The data are the mean \pm SD of at least three independent experiments, each performed in triplicates.

Results and discussion

Uptake and photocytotoxicity of the cyclodextrin conjugate

For easy and direct comparison, the photocytotoxic potential of CDFC was assessed using the same experimental procedure as that described in our previous work using tri-cationic porphyrins as photosensitizers.²² As a consequence, prior to irradiation, the CDFC uptake was measured after incubating the NCTC 2544 keratinocytes for 3 h with increasing concentrations of the photosensitizer in FCS-supplemented EMEM. Under these conditions, the CDFC uptake is practically proportional to the incubation concentration and the intracellular concentration is quite similar to that measured with the tri-cationic 5-(4-carboxyphenyl)-10,15,20-tris(4-methylpyridinium)-porphyrin poly-S-lysine conjugate (P-(Lys)_n) (Fig. 2A).

The CDFC phototoxicity estimated by the NR uptake assay was also compared to that of P-(Lys)_n. After incubation with 1 to 5 μ M CDFC or 5 μ M P-(Lys)_n in FCS-supplemented EMEM, NCTC 2544 keratinocytes were irradiated with increasing light doses (Fig. 2B). Control experiments were performed with cells incubated with CDFC but sham-irradiated (up to 60 min). No alteration of the NR uptake was observed with these controls as compared to native cells. Moreover, CDFC exhibits no cytotoxic effect in the dark for incubation concentrations up to 5 μ M (data not shown). It turns out that CDFC is as effective a photosensitizer

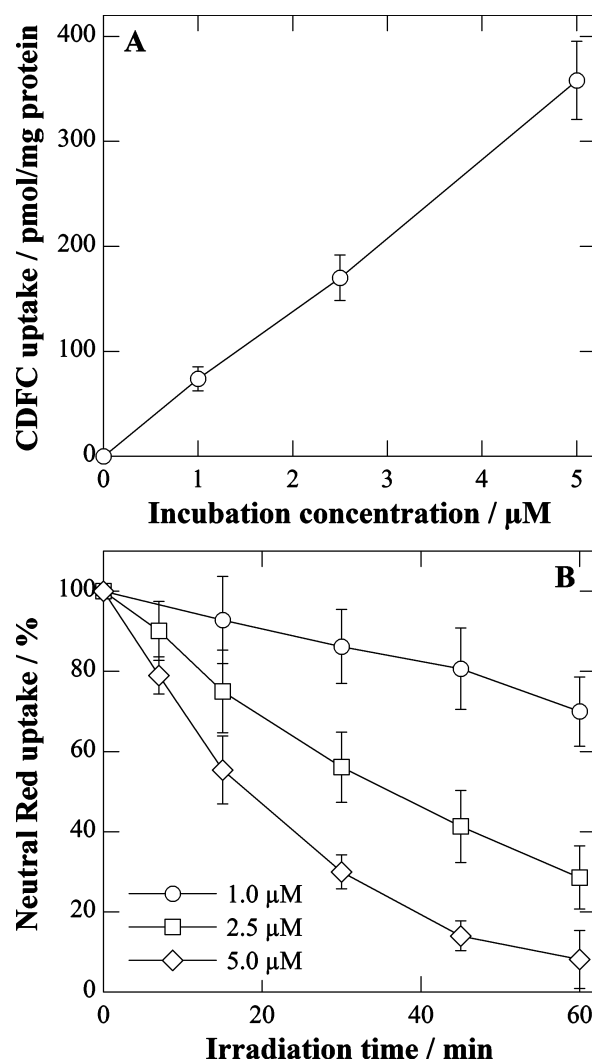


Fig. 2 (A) Uptake of CDFC as a function of the incubation concentration. Cells were incubated for 3 h with the desired chlorin concentration in FCS-supplemented EMEM. Data are the mean of at least three independent experiments performed in triplicates. (B) Cytotoxic effects photosensitized by CDFC as a function of the irradiation time and of the incubation concentration. Prior to irradiation, cells were incubated for 3 h with CDFC in FCS-supplemented EMEM and then irradiated for 30 min. A Neutral Red uptake of 100% corresponds to sham-irradiated untreated cells. Data are the mean of at least three independent experiments performed in triplicates.

as our reference P-(Lys)_n. Thus \sim 40% survival was observed with both CDFC and P-(Lys)_n after incubation with 5 μ M of the photosensitizers and irradiation for 30 min.

In view of this excellent *in vitro* photobiological activity, the photophysical properties of the cyclodextrin conjugate and the effect of the conjugation on the excited properties of the FC chlorin ring were studied.

First singlet excited state properties of FC and CDFC

Steady state results. The absorbance spectra of CDFC in ethanol, buffer or in TX100 micelles show the characteristic features of the chlorins with the strong Soret band with maximum absorbance at \sim 400 nm and the four Q bands with the more

pronounced first Q band ($\epsilon \sim 20\,000\text{ M}^{-1}\text{ cm}^{-1}$ at $\sim 650\text{ nm}$) (Table 1). The substitution of a fluorine atom at the *para* position on the four phenyl rings of FC by a β -cyclodextrin has only small effects on the position of the wavelengths of maximum absorbance of CDFC in all the solvents but the molar absorbance is notably decreased in plain buffer as compared to ethanol (Fig. 3A). However, a higher molar absorbance is restored by addition of TX100. The fluorescence spectrum and the fluorescence quantum yield ($\Phi_F \sim 0.25$) of the CDFC derivative are practically unchanged in going from ethanol to buffered aqueous solution (Fig. 3B). On the other hand, addition of TX100 leads to $\Phi_F = 0.5$ for CDFC, a value comparable to that of FC in ethanol and TX100. Whereas interaction of TX100 in the monomeric state with the cavity of β -cyclodextrin cannot be excluded for CDFC, in the case of FC, the phenoxy poly (oxyethylene glycol) groups favor hydrophobic ring–ring interaction with the chlorin ring and solubilisation of FC in the micellar environment.²⁴ Due to its limited solubility in buffer ($\sim 1\ \mu\text{M}$), FC tends to rapidly aggregate leading to broadening of all absorption bands with strong decrease in absorbance and to a red shift and a strong quenching of the fluorescence. On the other hand, FC, in contrast to CDFC, is readily soluble in a non-polar solvent such as toluene where it strongly fluoresces ($\Phi_F = 0.61$, Table 1).

The CDFC fluorescence decay by two parallel monomolecular processes in ethanol may be explained by the presence of two structurally different molecular arrangements. It may be supposed that in such structures, the chlorin FC macrocycle is “engaged” in three-dimensional networks created by the four β -cyclodextrin rings around the chlorin with its bulky benzyl-isoxazolidine ring.

In buffer, only the long-lived component is observed suggesting that in this more polar solvent, a single positioning of the four β -cyclodextrin moieties around the chlorin FC macrocycle is imposed by the interaction of the strongly polar water molecules with the hydrophobic chlorin ring. Distinct properties of singlet excited CDFC suggesting special arrangement of the β -cyclodextrins in ethanol and buffer are supported by femtosecond spectroscopy.

Ultrafast spectroscopy results. Time resolved ultrafast (femtosecond) absorption spectroscopy from the lowest excited singlet state (S_1) of FC and CDFC is of practical interest since it may unravel specific interaction of the β -cyclodextrin moieties with the chlorin ring in the S_1 state. Being given that the fluorescence lifetimes of FC and CDFC are of the order of several nanoseconds, there exists a spectroscopic window extending from light absorption to several hundred picoseconds in which a quasi-stable S_1 state is reached allowing study by appropriate technology of transient absorption from the S_1 state to upper excited singlet states (S_n) of the molecules. Thus, in principle the study of excited singlet states by ultra-fast spectroscopy is comparable to that of the well-known absorption spectroscopy of the triplet states.

Fig. 4A shows the transient absorbance changes observed with CDFC in buffer and ethanol at short and “long” time after excitation with 0.13 ps, 387 nm laser light pulses. The strong negative absorbance change in the 650–680 nm region is mainly due to the fluorescence of CDFC (see Fig. 3B) with some contribution of the bleaching of the S_0 CDFC absorbance. In this regard, the $\sim 50\%$ drop recorded within 1.5 ns in buffer (Fig. 4A) and in ethanol (data not shown) cannot be explained on

Table 1 Absorbance and fluorescence parameters of *meso*-tetrakis(pentafluorophenyl)-chlorin (FC) and *meso*-[tetra- β -cyclodextrin-(tetra-fluorophenyl)]-chlorin (CDFC) in various solvents

Solvent	CDFC					FC				
	Absorbance		Fluorescence			Absorbance		Fluorescence		
	$\lambda_{\text{max}}^a/\text{nm}$	$\epsilon^a/\text{M}^{-1}\text{ cm}^{-1}$	$\lambda_{\text{max}}^a/\text{nm}$	Φ_F^a	τ_F^a/ns	$\lambda_{\text{max}}^a/\text{nm}$	$\epsilon^a/\text{M}^{-1}\text{ cm}^{-1}$	$\lambda_{\text{max}}^a/\text{nm}$	Φ_F^a	τ_F^a/ns
Ethanol	407	135 000	649	0.23	1.39 (20.2%) 7.46 (79.8%)	400	182 100	649	0.51	7.3
	502	13 300				501	17 600			
	528	5 200				526	4 100			
	591	4 600				594	4 600			
	645	24 000				646	42 500			
pH 7 buffer ^b	404	98 300	648	0.27	8.07	401	99 300 ^d	661	0.0011 ^d	
	505	9 400				502	24 600 ^d			
	529	3 600				601				
	598	3 000				654				
	644	22 800								
Triton X100 ^c	409	122 900	649	0.50	6.30	405	176 700	656	0.55	
	503	11 400				503	19 600			
	531	4 200				530	4 400			
	592	3 500				595	5 300			
	646	23 000				650	46 400			
Toluene						407	162 500	653	0.61	
						504	17 300			
						532	12 600			
						597	12 100			
						650	43 200			

^a λ_{max} : wavelengths of maxima of absorbance and fluorescence; ϵ : molar absorbance; Φ_F : fluorescence quantum yield; τ_F : fluorescence lifetime. ^b 10 mM phosphate buffer. ^c 0.3% Triton X100 in pH 7, 10 mM phosphate buffer. ^d Unstable solution due to poor solubility.

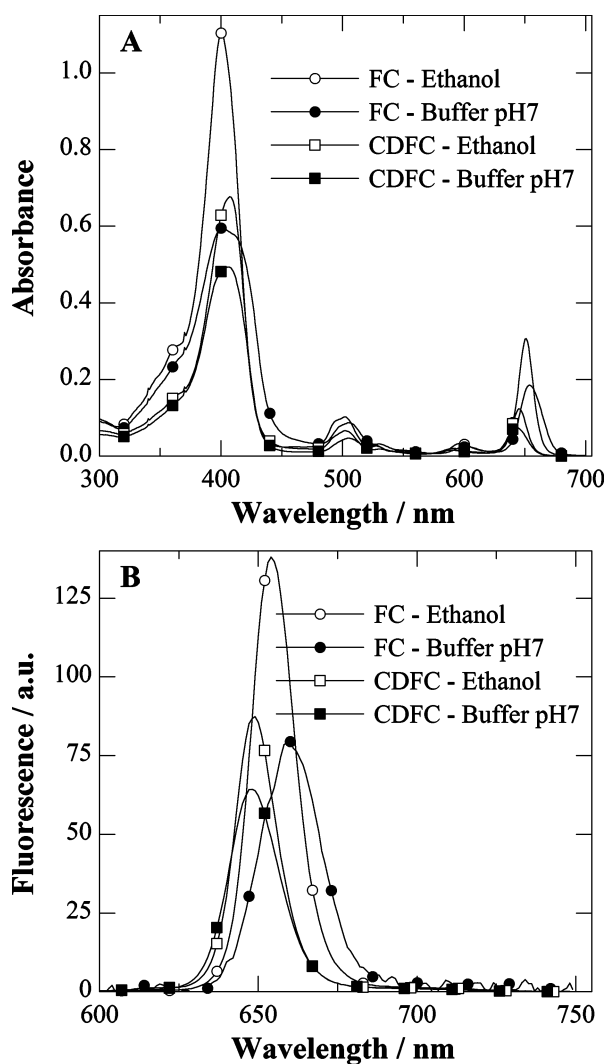


Fig. 3 (A) Absorbance spectra of FC (●)(○) and CDFC (□)(■) in pH 7, 10 mM buffer (●)(■) and in ethanol (○)(□). (B) Fluorescence spectra of FC and CDFC in pH 7, 10 mM phosphate buffer and in ethanol. Symbols are the same as in (A). The excitation wavelength was 400 and 407 nm for FC and CDFC, respectively. The absorbance of the solutions (light path: 1 cm) was ≤ 0.05 in all the cases. The sensitivity of the detection was increased 100-fold for FC in buffer. The temperature was 23 °C.

the basis of the fluorescence lifetimes reported in Table 1. Positive absorbance changes are observed in the visible wavelength range and their time-dependence at 475 nm is presented in Fig. 4B. With the CDFC derivative, the S_1S_n absorption is considerably reduced (about 2/3) *via* a monomolecular process (rate constant $\sim 5 \text{ ps}^{-1}$) which parallels the fluorescence drop of Fig. 4A, an almost constant fluorescence being observed at times greater than 30 ps (data not shown). On the other hand, a comparable decay is not observed with FC (Fig. 4B). This observation is consistent with an interaction of the β -cyclodextrins with the chlorin ring perturbing the CDFC S_1 state and leading to the Φ_F drop observed in ethanol and buffer with CDFC (Table 1). Thus the radiative energy loss may possibly be due to increased non-radiative internal conversion and/or to reaction of the S_1 state with proximal OH groups of the four β -cyclodextrins and/or solvent molecules encaged in the CD cavities whose local concentration is by force quite high.

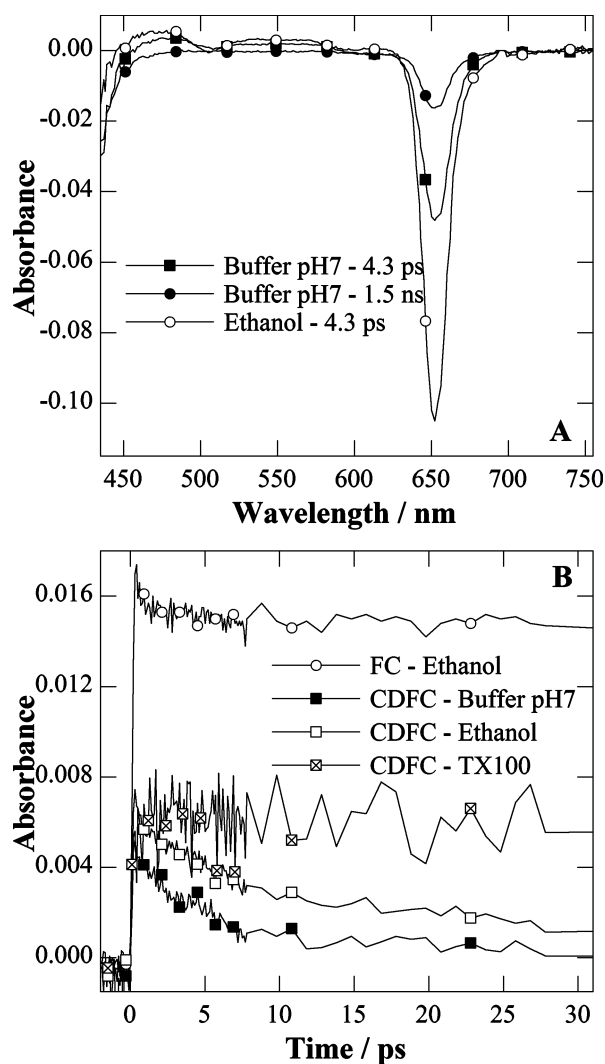


Fig. 4 (A) Transient difference absorbance spectra obtained 4.3 ps (■)(○) and 1510 ps (●) with femtosecond 387 nm laser flash photolysis of CDFC in air-saturated pH 7, 10 mM phosphate buffer (■)(●) and ethanol (○). (B) Decays of the transient absorbance at 475 nm after femtosecond 387 nm laser flash photolysis of CDFC in pH 7, air-saturated 10 mM phosphate buffer (■), ethanol (□) or TX100 (⊗) shown in Fig. 2B and of FC in ethanol (○).

In this regard, slight modification of the CDFC ground state absorbance spectrum is observed after laser flash experiments. Interestingly, when CDFC is incorporated into TX100 micelles, the fluorescence lifetime (Table 1) remains close to those measured in buffer and ethanol but a much stable S_1S_n absorption (Fig 4B) and a much higher Φ_F (Table 1) are observed. These changes suggest an important conformational reorganization induced by the TX100 micelles whose relatively small molecular size, *e.g.* $\leq 10.5 \text{ nm}$, imposes strong molecular constraints impeding the reorganization of the cyclodextrin “cage” and the interaction with the chlorin ring.²⁵

Triplet state properties of FC and CDFC

Porphyryns and chlorins are archetypes of the so-called photodynamic agents. They are recognized as type II photodynamic agents through activation of dioxygen molecules by energy transfer

from their lowest excited triplet state (3T_1) to oxygen triplet ground state to form singlet oxygen (1O_2). Type I electron transfer reactions although much less frequent are also possible because tetrapyrroles are electron donors to strong electrophiles such as nitroimidazoles.²⁶ As a result, it is essential to characterize the triplet state properties of a potential PDT photosensitizer such as CDFC. Thus, transient absorbance spectrum, quantum yield of 3T_1 formation and rate constant of interaction of 3T_1 with oxygen are the most relevant parameters which may help to understand its interesting photobiological activity. Fluorescence lifetimes of the order of 10 ns (Table 1) suggest that the triplet states of FC and CDFC are populated with a rate constant of $\sim 10^8$ s $^{-1}$ consistent with a $S_1(\pi\pi^*) \rightarrow T_1(\pi\pi^*)$ process.

Triplet state transient spectra. Fig. 5A shows the transient difference absorbance spectra of CDFC measured at various times after 355 nm nanosecond laser flash spectroscopy of CDFC in de-

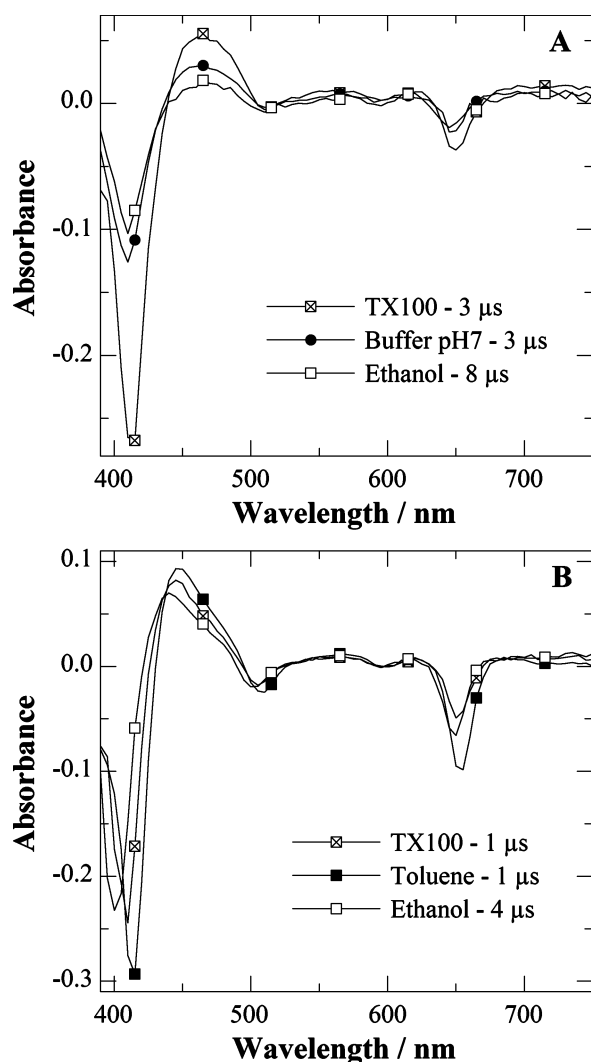


Fig. 5 (A) Transient difference absorbance spectra obtained 3 μ s (●)(□) and 8 μ s (□) after 355 nm laser flash photolysis of 16 μ M CDFC in pH 7, 10 mM phosphate buffer (●), TX100 (□) and ethanol (□). All the solutions were saturated with N_2 . (B) Transient difference absorbance spectra obtained 1 μ s (□)(■) and 4 μ s (□) after 355 nm laser flash photolysis of 12 μ M FC in toluene (■), TX100 (□) and ethanol (□). All the solutions were saturated with N_2 .

aerated buffer, ethanol and TX100. It can be seen that the spectral shapes are practically independent of solvents and time delays after the laser pulse. All the transient species show a strong broad absorbance with maximum around 460 nm and minor maxima at about 560, 620 and 720 nm corresponding to transitions between 3T_1 to higher triplet levels 3T_n , as generally observed with this class of molecules.^{27,28} The negative absorbance observed in the absorbance region of the Soret and main Q bands results from the depopulation of ground state molecules by the exciting laser flash to produce 3T_1 molecules.

The decay of these transient triplet absorptions strongly depends on solvent. While the CDFC triplet slowly decays in de-aerated TX100 micellar solution by a simple first order process with a lifetime of 215 μ s (Table 2), more complex kinetics are observed in ethanol and water (Fig. 6). In these two last solvents, a fast initial decay yields a longer lived species whose spectral characteristics (Fig. 5A) are apparently the same as those of the short-lived components. The first order decay of these long-lived transients apparently leads to partial recovery of the ground state absorbance of Soret and first Q bands (Fig. 6) supporting the contention that these species have structural chemical features similar to the ground state CDFC. A major difference between the longer lived species of CDFC in ethanol and in the buffered solution is that in the aqueous medium the longer lived species lifetime is 427 μ s while it is only 1.2 μ s in ethanol in which only partial recovery of the CDFC ground state absorbance is observed after the triplet molecules have decayed. However femto- and nano-second laser spectroscopy data do not make it possible to conclude whether in ethanol the photochemistry involves upper singlet states or the triplet state of CDFC.

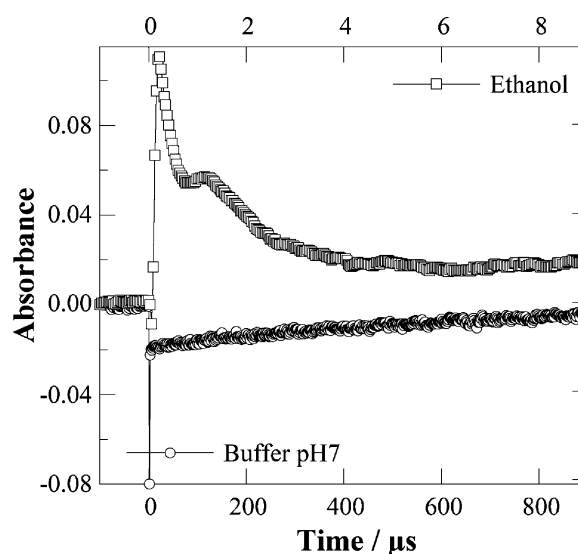


Fig. 6 Decay of the transient absorbance at 460 nm in N_2 -saturated solutions after nanosecond 355 nm laser flash photolysis of 16 μ M CDFC in ethanol (□) and recovery of absorbance at 650 nm in pH 7, 10 mM phosphate buffer (○).

The contribution of the four β -cyclodextrins to the triplet state properties of CDFC is evidenced by the study of FC whose difference absorbance spectra after laser flash excitation under various solvent conditions are presented in Fig. 5B. While the bandwidth of the difference absorbance band of FC in the

Table 2 Triplet state parameters of *meso*-tetrakis(pentafluorophenyl)-chlorin (FC) and *meso*-[tetra- β -cyclodextrin-(tetra-fluorophenyl)]-chlorin (CDFC) in various solvents at 25 °C

FC					CDFC			
Solvent	$\lambda_{\max}^a/\text{nm}^a$	$\epsilon^a/\text{M}^{-1}\text{cm}^{-1}$	$\tau_T^a/\mu\text{s}$	$k(\text{O}_2)^a \times 10^{-9}/\text{M}^{-1}\text{s}^{-1}$	$\lambda_{\max}^a/\text{nm}^a$	$\epsilon^a/\text{M}^{-1}\text{cm}^{-1}$	$\tau_T^a/\mu\text{s}$	$k(\text{O}_2)^a \times 10^{-9}/\text{M}^{-1}\text{s}^{-1}$
Ethanol	440	50 000	141 \pm 1	1.3 \pm 0.1	465	26 000 ^d	0.28 \pm 0.01 ^d 1.18 \pm 0.04 ^d	2.2 \pm 0.3 ^d
pH 7 buffer ^b					462	35 000 ^d	2.4 \pm 0.6 ^d 427 \pm 5 ^d	1.9 \pm 0.2 ^d
Triton X100 ^c	446	53 000	247 \pm 2	2.0 \pm 0.2 ^c	465	32 000	215 \pm 1	0.61 \pm 0.05 ^c
Toluene	444	42 000 32 000 ^f	249 \pm 2					

^a λ_{\max} : wavelength of maximum absorbance of difference transient absorption spectrum; ϵ : molar absorbance of triplet at this maximum estimated by method B unless otherwise stated; τ_T : triplet lifetime in de-aerated solution; $k(\text{O}_2)$: bimolecular reaction rate constant for triplet quenching by oxygen. ^b 10 mM phosphate buffer and 0.15 M NaCl. ^c 0.3% Triton X100 in pH 7, 10 mM phosphate buffer and 0.15 M NaCl. ^d Two species (see text), ϵ and $k(\text{O}_2)$ hold for the longer lived species. ^e Assuming O_2 -solubility to be the same as in buffer (1.2 mM at 20 °C). ^f Method A.

440–500 nm region with a maximum at about 450 nm, is narrower than that of CDFC, the wavelengths of the secondary maxima in the visible region are rather similar for the two derivatives. In contrast to CDFC, the FC transient absorptions decay by simple first order processes characterized by rather long lifetimes in all the solvents (Table 2).

The triplet nature of all these species is supported by their quenching by oxygen in O_2 - and air-saturated solutions which follows pseudo first order kinetics. However, the smaller reaction rate constant $k(\text{O}_2)$ obtained with the CDFC derivative in TX100 micelles suggests that the bulky cyclodextrin assembly in the chlorin ring vicinity intervenes in the quenching process (Table 2).

Determination of triplet molar extinction coefficients. Triplet identification was further ascertained using the $^3\text{T}_1$ state (^3FC) of a 12 μM FC solution in toluene as an energy donor to β -carotene (β -car) whose low-lying $^3\text{T}_1$ state energy is $\sim 7500\text{ cm}^{-1}$. Direct population of the β -carotene triplet ($^3\beta$ -car) is negligible since the intersystem crossing yield is exceedingly small.²⁹ However the β -car $^3\text{T}_1$ state can be populated by energy transfer from molecules with $^3\text{T}_1$ states of higher energy. By this means, using the so-called comparative method, a molar extinction coefficient of $^3\beta$ -car has been determined to be $\sim 100\,000\text{ M}^{-1}\text{ cm}^{-1}$ at 520 nm. This wavelength has two advantages since it is close to the absorbance maximum of $^3\beta$ -car in toluene, and the β -car ground state molar absorbance at 520 nm is only $1\,900\text{ M}^{-1}\text{ cm}^{-1}$.^{30,31} Assuming a transfer efficiency of unity from the long-lived FC triplet state to the β -car triplet, the concentration of molecules in the first excited FC triplet state can be obtained from which molar extinction coefficients are measured (method A). By this method, the molar extinction coefficient of triplet FC at 450 nm, the wavelength of maximum absorbance, can be estimated to be $\sim 32\,000\text{ M}^{-1}\text{ cm}^{-1}$ (see ESI†).

Another method (method B) allowing an estimate of the ϵ ($^3\text{T}_1$) triplet FC is based on the determination of the ratio (α) of the FC molecules in the $^3\text{T}_1$ state [^3FC] to the total ground state [FC_0] concentration. At any time (t) after the laser pulse and at any wavelength (λ), the transient difference absorbance $\Delta\text{Abs}(t)$ must be for simple first order processes: $\Delta\text{Abs}(t) = \text{Abs}(^3\text{FC})(t) - \alpha \times \text{Abs}(\text{FC}_0)$.^{27,32} With this method, ϵ of ^3FC

at 450 nm is found to be $42\,000\text{ M}^{-1}\text{ cm}^{-1}$, in fair agreement with the value estimated by the energy transfer method (see ESI†). Thus, an average value of $35\,000\text{ M}^{-1}\text{ cm}^{-1}$ would be realistic being given the uncertainty inherent to such determinations. Molar extinction coefficients estimated using method B are given for both FC and CDFC in all the studied solvents. Overall, it can be seen that these values are smaller for CDFC. However, they are consistent with molar extinction coefficients previously reported for *meso*-tetrakis(*meta*-hydroxyphenyl)chlorin (Foscan®) and glucoconjugated derivatives embedded in cyclodextrins.²⁷

Triplet quantum yield of FC and CDFC. Knowing the molar absorbance of the FC $^3\text{T}_1$ state, it is possible to estimate the $^3\text{T}_1$ formation quantum yield (Φ_T) by the comparative method.³¹ The *meso*-tetraphenylporphyrin (TPP) was used as an actinometer since its Φ_T is 0.8 in toluene and $\Delta\epsilon = \epsilon(^3\text{T}_1) - \epsilon(^1\text{S}_0) = 35\,000\text{ M}^{-1}\text{ cm}^{-1}$ at 450 nm.^{18,27} To this end a solution of TPP with an absorbance corresponding to that of 12 μM FC at 355 nm was prepared in toluene to ensure equal population of singlet states of FC and TPP under excitation with the same laser energy.³¹ Considering that saturation effects are negligible (see ESI†), the Φ_T of FC can be easily calculated as 0.2 based on the maximal difference transient absorbance of TPP and FC triplet and the $\Delta\epsilon$ value of FC ($38\,700\text{ M}^{-1}\text{ cm}^{-1}$) at 450 nm.³¹ Fig. 5B suggests that the Φ_T value of 0.2 is probably valid for the other media in view of the spectral similarities of FC triplet in the studied solvents. As to CDFC, its insolubility in toluene and the complex triplet behavior in ethanol preclude any direct Φ_T determination. However, using the FC triplet as an actinometer for the CDFC triplet in TX100 (Fig. 5A), it can be suggested that the Φ_T of CDFC in this solvent is about the same as that of FC taking into account the lower molar extinction coefficient of triplet CDFC in TX100 (see Table 2). It can be seen that in all the cases $\Phi_T + \Phi_F < 1$, suggesting the intervention of internal conversion processes in the deactivation of the excited molecules in support to ultra-fast spectroscopic data.

Quantum yield of $^1\text{O}_2$ formation in buffered aqueous solution

The formation of a long-lived CDFC triplet state (Fig. 6), quenched by dioxygen at diffusion controlled rate (Table 2) in

buffered aqueous solutions, suggests the production of $^1\text{O}_2$. The $^1\text{O}_2$ generation was detected using His as a specific $^1\text{O}_2$ probe since its photosensitized oxidation occurs *via* a type II photodynamic mechanism in buffered aqueous solutions.^{19,26} The quantity of $^1\text{O}_2$ produced was assessed using a comparative method with *meso*-tetrasulfonatophenyl porphyrin (TPPS₄) as a reference water-soluble photosensitizer for which the quantum efficiency of $^1\text{O}_2$ generation in pH 7 buffer has been reported to be 0.6.³³ The 365 nm light dose-dependence of His consumption which, at low light doses follows first order kinetics, has been monitored by HPLC in solutions containing TPPS₄ or CDFC or FC as photosensitizer. The His consumption rate constants normalized to the fraction of absorbed light ($1 - 10^{-\text{Abs}}$, Abs being the absorbance of the solution at 365 nm) are proportional to the quantum efficiency of $^1\text{O}_2$ generation. Assuming $\Phi(^1\text{O}_2) \sim 0.6$ for TPPS₄, it turns out that $\Phi(^1\text{O}_2) \sim 0.4$ and <0.05 for CDFC and FC, respectively. Thus, although the Φ_T of CDFC could not be directly determined in buffer and being given the uncertainties inherent to the comparative methods used for Φ_T (see above) and $\Phi(^1\text{O}_2)$ determination, it may be proposed that the quenching of the CDFC triplets by dioxygen generates $^1\text{O}_2$ with high efficiency. On the other hand, the negligible $^1\text{O}_2$ generation by FC is readily understandable since in buffer, FC rapidly aggregates and precipitates (see section on first singlet excited state properties).

Conclusions

The goals of this work was to synthesize an effective water-soluble chlorin photosensitizer for which or other members of the same family easy formulation for routine photo-dermatology applications is granted. It appears that up to four β -cyclodextrins can be conjugated to a bulky *meso*-tetrakis(pentafluorophenyl)-chlorin with the reduced pyrrole ring linked to an isoxazolidine ring giving a water-soluble photosensitizer effective towards cultured proliferating human keratinocytes. The cell-penetrating property of the photosensitizer is an indispensable prerequisite in topical PDT. Cyclodextrins enhance topical drug permeability by keeping hydrophobic drug molecules in solution and delivering them to the skin, where they can interact with lipophilic cell components of the skin after disruption of the barrier function.^{34,35} Moreover, for topical PDT, skin lesions are usually submitted to prior curettage which decreases the *stratum corneum* barrier properties thereby possibly enhancing skin penetration of the conjugate.

This work demonstrates that the first excited triplet state of such derivatives can be highly populated in a variety of microenvironments thereby generating $^1\text{O}_2$, a major cytotoxin. Accordingly, an obvious and immediate development of this work, owing to the fairly good fluorescence of the fluorinated chlorin ring, will be the study of the time-dependent intracellular localization of the conjugate in cultured living cells by micro(spectro)fluorometric techniques. The full or partial removal of β -cyclodextrins through slow hydrolysis of the ether bonds by O-alkyl cleavage enzymes may be an effective way of modulating the intracellular localization/partition of the chlorin core within hydrophilic cytosolic sites and cell membranes, thus developing an effective photo-oxidative stress at multiple sites. Such a study is a prerequisite for a further extension of this work to skin tumor bearing animals.

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