Effect of Cyclodextrins on the Physicochemical Properties of Chlorophyll *a* in Aqueous Solution

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The interactions between chlorophyll *a* and two β-cyclodextrins, that have the same cavity size but different substituents, were studied in aqueous solutions. These supramolecular host−guest complexes were examined by a combination of UV/vis absorption, circular dichroism, NMR, and steady-state and time-resolved fluorescence measurements. The results indicate that all cyclodextrins solubilize the pigment mainly in monomeric form in water. The pigment forms 1:1 complexes with the heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin and 1:2 complexes with the hydroxypropyl-β-cyclodextrin. In such complexes the methyl groups of the cyclodextrin inner cavity are involved in the interaction with the pigment as evidenced by NMR measurements. We also measured the luminescence of singlet oxygen photosensitized by chlorophyll *a* in the inclusion complexes.

Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides constituted by a variable-number of α-D-glucopyranose residues by *O*-glycoside bonds of types 1−4, where the anomeric carbon C1 of one residue is bound to carbon C4 of the next residue.[1,2] The most useful property of CDs is their ability to form inclusion complexes with a large number of organic molecules.[3] This unique property has made the CDs extensively used in pharmacological, chemical, and many other applied areas.[4] Recently, in our laboratory a systematic study of the combined use of natural chlorine, chlorophyll *a* (Chl *a*), and chlorophyllide *a* (Chlide *a*), with CDs as potential sensitizers in photodynamic therapy (PDT)[5−8] was carried out. The advantage in the use of these natural chlorine resides on the fact that these pigments are characterized by intense absorption bands in the 600−850 nm wavelength region, where the maximal depth of light penetration into tissues is obtained.[9] In the present work, the effect of two β-CDs, hydroxypropyl-β-cyclodextrin (HP-β-CD) and heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD), on the physicochemical properties of Chl *a* has been investigated in aqueous solution using ground-state absorption, circular dichroism, NMR, and steady-state and time-resolved fluorescence measurements. Luminescence of singlet oxygen photosensitized by Chl *a* in the inclusion complexes is also reported.

Materials and Methods

Chl *a* was isolated from *Spirulina geitleri* and purified according to Iriyama et al.[10] HP-β-CD with a degree of substitution (DS) = 5.6 and TM-β-CD DS = 4.8, and all other solvents used were purchased from Aldrich and used without further purification. The samples were prepared as already reported.[7,8] Visible absorption spectra were recorded using a Varian CARY/3 spectrophotometer. Fluorescence and static resonance light scattering (RLS) measurements were carried out using a Varian Cary Eclipse fluorescence spectrophotometer. Fuorescence spectra were obtained by excitation at the maximum absorption of Chl *a* in the Soret band, while RLS spectra were recorded with an excitation neutral density filter with 6% of transmittance. The circular dichroism spectra were recorded using a JASCO J810 spectropolarimeter, and the fluorescence decays were obtained by time-correlated single-photon counting. A pulsed Ar^+ laser mode-locked (λ = 514 nm) was used as excitation source for the fluorescence lifetime measurements, with a repetition rate of 82 MHz and pulse duration of 150 ps. A synchronous dye (rhodamine) laser converts the incoming beam to $\lambda \sim 580$ nm and pulse duration to 2 ps. The power of the polarized (Glan-Taylor polarizer prism) laser beam on the sample is about 60 mW. The fluorescence emission is collected (at 90° through a monochromator) by a "microchannel plate" photomultiplier having a response of about 200 ps. The ^{1}H and ^{13}C NMR spectra were recorded on a 500 MHz Bruker spectrometer. The singlet oxygen quantum yields were determined according to Arbogast and Foote.[11] The measurements were carried out at room temperature.

Results and Discussion

The aggregation state of the pigment in aqueous solutions of cyclodextrins at different concentrations of Chl *a* and CDs was estimated by means of UV/vis absorption spectra.

Figure 1 shows the absorption spectra of Chl *a* in aqueous solution of TM-β-CD and HP-β-CD, respectively. For both Chl *a*/CDs complexes, there is a bathochromic shift of about 5 nm for the Soret band and 10 nm for the Q_y (0,0) transition with respect to the Chl *a* monomeric absorption spectra in ethyl ether. This shift, as previously reported for Chl *a*,[6,7] indicates the

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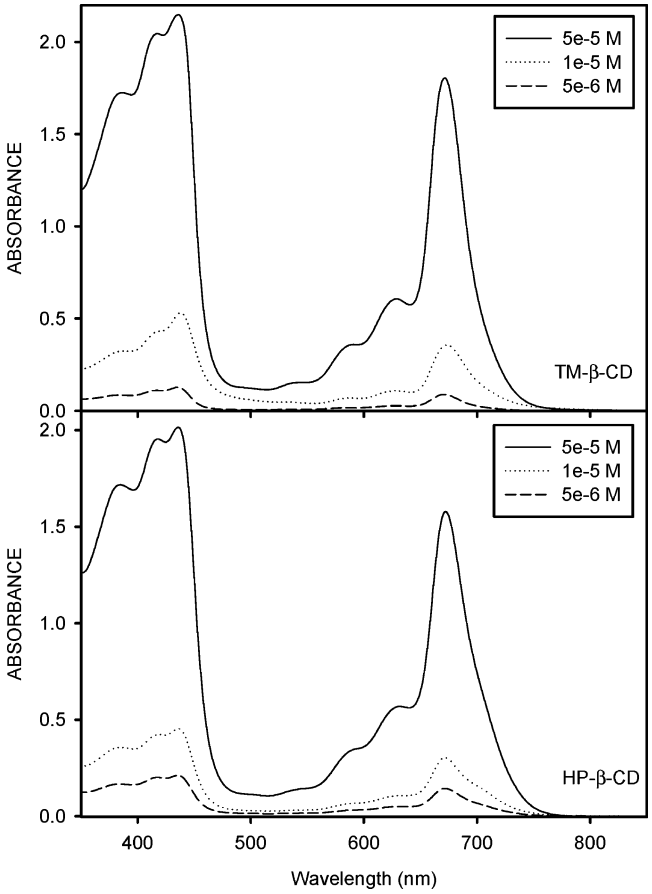


Figure 1. Absorption spectra of 0.1 M CD solution at different Chl *a* concentrations.

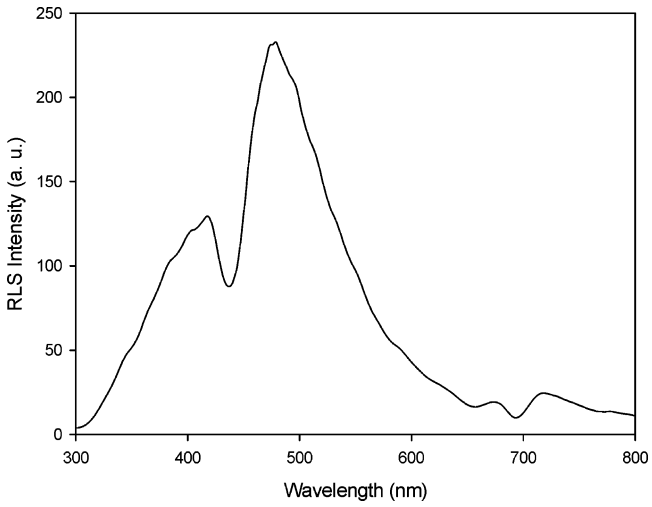


Figure 2. RLS profile of the 1×10^{-5} M Chl *a* in the presence of 0.1 M HP-*β*-CD.

formation of an inclusion complex of CDs with Chl *a*. In addition it is possible to observe an enlargement of the peak in the red region for both Chl *a*/CDs complexes. The absorption spectrum of 1×10^{-5} M Chl *a* in the presence of HP-*β*-CD shows an additional peak of low intensity in the red region (745 nm), which can be attributed to the presence of aggregated forms of Chl *a*[12] as confirmed by measurements of RLS (Figure 2). RLS is, in fact, a sensitive and selective probe to chromophore aggregation.[13] In particular the presence of a strong resonance scattering signal between 400 and 500 nm is indicative of large aggregates with a strong excitonic coupling among the chromophores.[14]

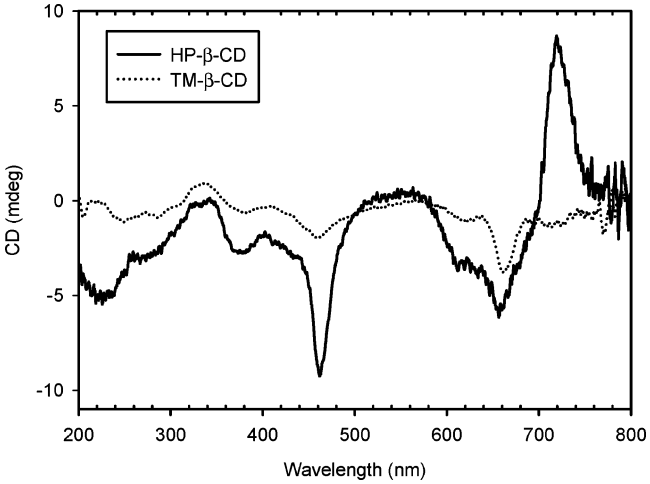


Figure 3. Circular dichroism spectra of 1×10^{-5} M Chl *a* in different 0.1 M CD solutions.

Figure 3 shows the circular dichroism of Chl *a* in HP-*β*-CD and TM-*β*-CD solutions. For the HP-*β*-CD, there is a nonconservative red band splitting characterized by a positive peak at about 730 nm and a negative one at about 660 nm. This splitting can be attributed to the presence of the aggregated form of Chl *a*.[14,15] In the case of the TM-*β*-CD, the circular dichroism spectrum suggests the presence of nonconservative peaks of low intensity at about 660 and 450 nm, very similar to the peaks that characterize the circular dichroism of Chl *a* monomer in ether,[16] thus confirming the presence of the pigment prevalently in monomeric form.

In Figure 4 are reported the emission spectra of Chl *a* (1×10^{-5} M) in aqueous solutions of HP-*β*-CD and TM-*β*-CD. It is possible to observe, as a general behavior, an increase of the fluorescence intensity of Chl *a* with increasing CD concentration. The same results were observed also at lower pigment concentration. This indicates that the inner filtering effects are negligible and that the increase of the fluorescence intensity evidences the formation of the monomer as a result of the inclusion processes. The emission intensity of Chl *a* in TM-*β*-CD appears always higher than in HP-*β*-CD. This phenomenon can be related to the presence in HP-*β*-CD solution of aggregated species of the pigment, also evidenced by the corresponding absorption spectra (Figure 1), able to quench the monomer fluorescence signals by dipole−dipole energy transfer, as already reported in the literature.[17] This behavior, which was observed at all the Chl *a* concentrations tested (data not shown), indicates a higher presence of the pigment aggregated form in HP-*β*-CD compared to TM-*β*-CD.

The binding constants for the inclusion complexes of Chl *a* with HP-*β*-CD and TM-*β*-CD were calculated by applying the modified Benesi−Hildebrand treatment to the fluorescence measurements in the following form:[18]

$$\frac{F^\circ}{F - F^\circ} = \frac{1}{A} + \frac{1}{AK[\mathrm{CD}]^n}$$

where K is the binding constant, F° is the initial fluorescence intensity of free Chl *a*, F is the maximum value reached by the fluorescence intensity of the Chl *a*−CD inclusion complex, A is a constant, and n is the number of binding sites. Due to the low solubility of Chl *a* in water, the F° value was obtained following complete solubilization of the pigment in a mixture of water and ethanol 80:20 (v:v). In these experiments, the

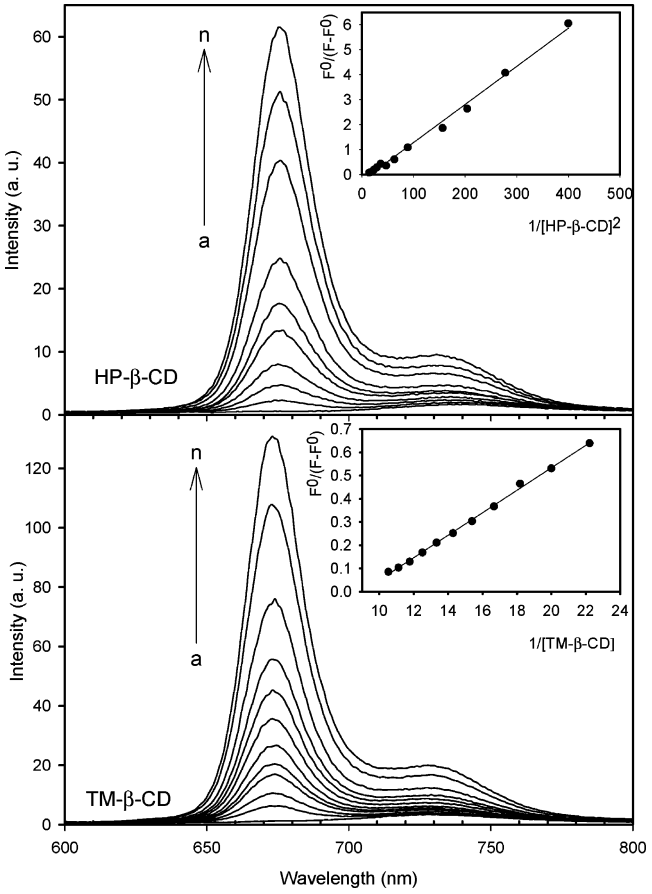


Figure 4. Fluorescence spectra of 1×10^{-5} M Chl *a* at different CD concentrations: [HP-*β*-CD] = 0.010 (a), 0.070 (b), 0.106 (c), 0.126 (d), 0.146 (e), 0.186 (f), 0.206 (g), 0.226 (h), 0.246 (i), and 0.266 M (l); [TM-*β*-CD]= 0.010 (a), 0.050 (b), 0.060 (c), 0.070 (d), 0.075 (e), 0.080 (f), 0.085 (g), 0.090 (h), 0.095 (i), 0.105 (l), 0.115 (m), and 0.120 M (n). Insets: Benesi−Hildebrand plots of $F°/F - F°$ vs 1/[CD]^n of Chl *a* in the presence of CD: $n = 1$ for HP-*β*-CD and $n = 2$ for TM-*β*-CD.

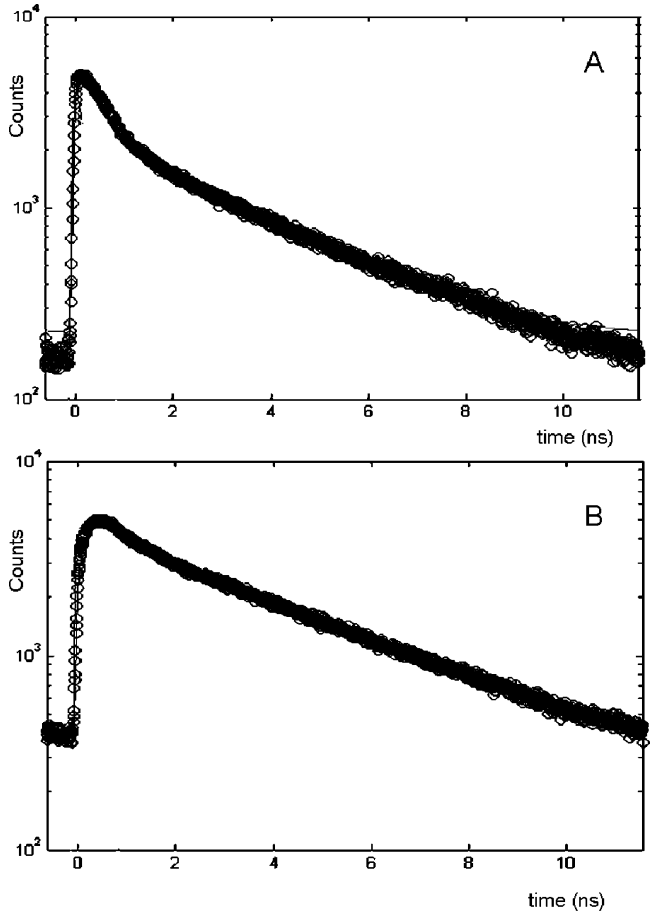


Figure 5. Fluorescence emission decay traces ($\lambda_{ex} = 430$ nm) of Chl *a* (1×10^{-5} M) in aqueous solution of different CDs (0.1 M): (A) HP-*β*-CD and (B) TM-*β*-CD.

concentration of Chl *a* was kept to 1×10^{-5} M, whereas the concentration of cyclodextrins varied from 3×10^{-2} to 2×10^{-1} M.

The inset of Figure 4 is the plot of $F°/(F - F°)$ vs 1/[HP-*β*-CD]^2. The linearity of the plot reflects the formation of a 1:2 complex between the Chl *a* and HP-*β*-CD. From the slope and intercept of the curve it is possible to calculate the binding constant *K*. The value obtained for HP-*β*-CD/Chl *a* was 15 ± 2 M^{-2}. In contrast, the linearity of the plot $1/(F - F°)$ vs 1/[TM-*β*-CD] reflects the formation of a 1:1 complex between the pigment and the TM-*β*-CD complex (inset Figure 4). Also in this case, the binding constant *K* was calculated from the slope and intercept; the value obtained was 9 ± 2 M^{-1}.

The inclusion complexes between Chl *a* and CD have been also studied by means of time-resolved fluorescence. Figure 5A shows the time-resolved fluorescence decay for the Chl *a* in HP-*β*-CD solution. The curve exhibits a double-exponential decay, with 45% of a slower phase ($\tau_1 = 76$ ps) and 55% of a faster phase ($\tau_2 = 2{,}3$ ns). Since lifetimes of about 60 ps and about 5 ns are reported for Chl *a* aggregates and monomer, respectively,[19] the results obtained suggest that the Chl *a* in HP-*β*-CD solution is present as monomer and aggregated form in comparable amounts.

Figure 5B shows the time-resolved fluorescence for the Chl *a* in TM-*β*-CD solution. In this case as well, the decay cannot be fitted by a single exponential and has two characteristic lifetimes: $\tau_1 = 0.26$ ns (19%) and $\tau_2 = 4.2$ ns (81%). This suggests that, also in this case, the Chl *a* is present both in monomeric and in aggregated form but that the monomer largely predominates. The slightly higher values calculated for Chl *a* in TM-*β*-CD compared to the corresponding values in HP-*β*-CD can be reasonably ascribed, in agreement with the literature,[20] to the different environment of the two CDs.

To get further information on the dynamics of these supramolecular systems, we performed time-resolved fluorescence polarization anisotropy experiments. The fluorescence decay traces exhibit an exponential behavior, and they have been analyzed according to the following equation:

$$r(t) = r(0) \exp(-t/\tau_r) \qquad (1)$$

where $r(0)$ is the polarization anisotropy at time zero and τ_r is the rotational correlation time. The best fitting procedure of the experimental data with eq 1 gave the values of 2.6 and 2.1 ns for Chl *a*/TM-*β*-CD and Chl *a*/HP-*β*-CD, respectively. Because the rotational correlation time is related to the size of the emitting system,[21] the comparison between our experimental results and the value of 0.25 ns found for monomeric Chl *a* in organic solvent[22] indicates a reduced mobility of the pigment in these solutions and therefore the formation of inclusion complexes.

The ^{1}H NMR spectrum of TM-*β*-CD showed small chemical shift changes in the presence of Chl *a* (data not shown). Significant chemical shift changes, instead, were observed in the ^{13}C NMR spectrum of the CD when the pigment was added to the solution. All carbons are deshielded (Table 1), and the

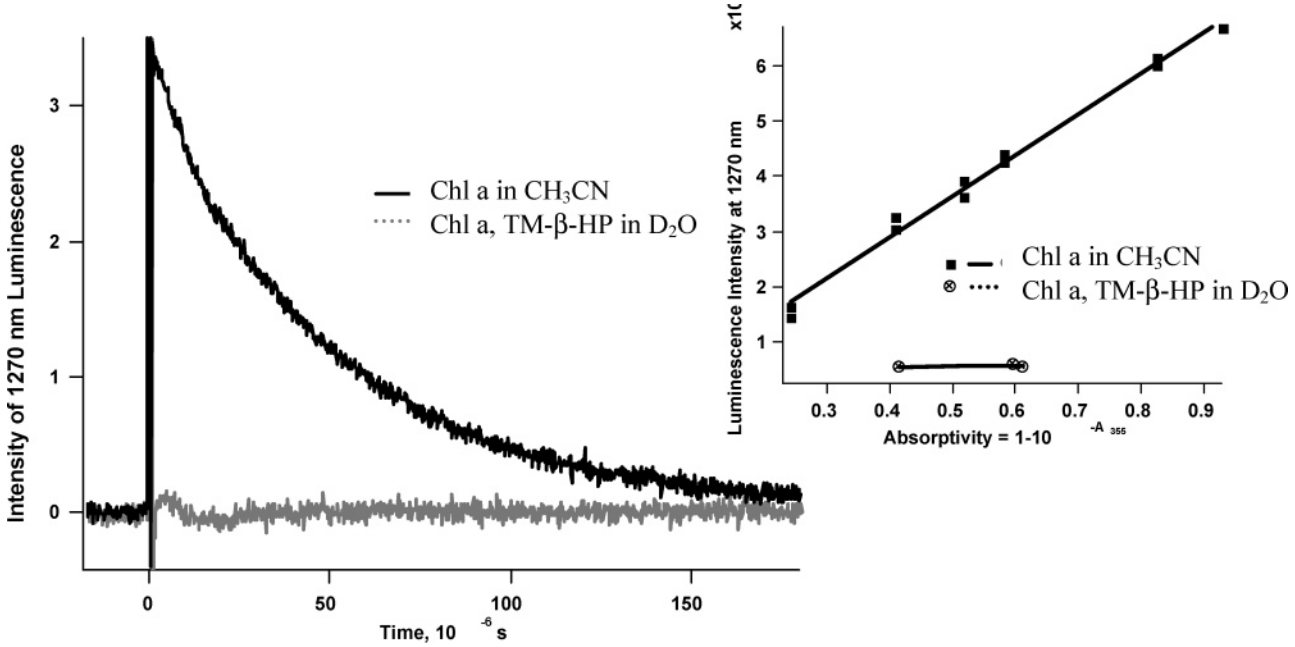
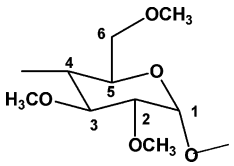


Figure 6. (A) Luminescence decay traces for A_{355}-matched solutions of Chl *a* in acetonitrile and Chl *a* of TM-*β*-CD in D_2O. (B) Quantum yield deteremination: the ratio of the slopes of the luminesacence plotted against absorptivity ($1 - 10^{-A_{355}}$) is the ratio of their quantum yields.

TABLE 1: Chemical Shift Change $\Delta\delta = \delta_{complex} - \delta_{free}$ of TM-*β*-CD in D_2O in the Presence of Chl *a* at 25 °C



	δ(0.1 M TM-*β*-CD)	δ(0.1 M TM-*β*-CD/ 5×10^{-5} M Chl *a*)	$\Delta\delta$
C1	96.72	99.44	2.72
C2	80.66	83.35	2.69
C3	79.76	82.45	2.69
C4	76.69	79.44	2.75
C5	70.49	73.80	3.31
C6	70.08	72.78	2.70
CH_3	59.44	62.17; 62.13	
CH_3	58.07	60.79; 60.76	
CH_3	57.80	60.51; 60.47	

methenyl groups of the cyclodextrin inner cavity are split into two different signals, demonstrating that the interaction with the pigment involves the inner cavity. Similar results have been reported for the ^{13}C NMR spectrum of the TM-*β*-CD complex with (+)- and (−)α-pinene.[23] ^{13}C NMR spectra of 10^{-1} M HP-*β*-CD show enlarged signals attributable to the presence of cyclodextrin aggregates in solution.

Because of this signal enhancement, the differences caused by the presence of 5×10^{-5} M Chl *a* were less evident than in TM-*β*-CD, and the cyclodextrin gives a smaller chemical shift (0.01−0.02 ppm).

Since the TM-*β*-CD complex allows solubilization of Chl *a* prevalently in monomeric form, preliminary singlet oxygen luminescence measurements have been performed on this sample (Figure 6). The standard used was Chl *a* in acetonitrile (CH_3CN) with a quantum yield (ϕ_Δ) of 0.60. The indexes of refraction of the two solvents, CH_3CN and D_2O, were almost identical (1.34 vs 1.33), so no further corrections were made. The quantum yield of TM-*β*-CD is 0.076 (relative to Chl *a* in CH_3CN). Surprisingly the solutions showed only minimal singlet oxygen luminescence. This result, however is close to that previous reported[6] and shows that generally the CD/Chl *a* complexes need a better investigation of their photophysical properties.

The results obtained with the different techniques on the effect of CDs on the physicochemical properties of Chl *a* in aqueous solution indicate that the HP-*β*-CD and TM-*β*-CD solubilize the pigment mainly in monomeric form in water, forming inclusion complexes. The complex binding constant values are of the same order of magnitude as those reported in the literature for CD/alkyl complexes,[24] suggesting that in CD/Chl *a* complexes the interactions are mainly hydrophobic between the phytyl chain of the pigment and the inner cavity of the CDs. The inclusion of the alkyl chain into the CD cavity, although entropically unfavorable, is known to be promoted by van der Waals and hydrophobic interactions between the chain and the CD as reported for PEG/CD (PEG = poly(ethylene glycol)) complexes.[25,26] The different stoichiometry of the complexes can be discussed in view of the different substitution of the CD functional groups. In particular for the HP-*β*-CD, the presence of a great number of nonsubstituted hydroxyl groups favors the CD aggregation, by means of hydrogen bond formation, as suggested by NMR data. As already reported,[25] the hydrogen bonding interaction between two CDs promotes the formation of PEG/HP-*β*-CD necklace complexes. Geometrical consideration on the length of the pigment phytyl chain (about 21 Å) and the cavity depth of a single CD (about 8 Å) suggest that two interacting HP-*β*-CDs can be threaded by the phytyl chain of one Chl *a* molecule, thus explaining the 2:1 stoichiometry of the inclusion complexes. The occurrence of further hydrogen bond interactions between different HP-*β*-CD/Chl *a* complexes, promoting the approach between pigment macrocycles, can result in the Chl *a* excitonic coupling observed in the circular dichroism and UV/vis spectra and can also justify the multiexponential fluorescence decay.

On the contrary, the presence of a minor number of hydroxyl groups on the TM-*β*-CD results in a lower tendency of this molecule to aggregate, as suggested by the well-defined and sharp signals of its NMR spectrum in solution. The interactions between the phytyl chain of the pigment and the inner cavity of the CD are evidenced by the splitting of NMR signals relative

to the CD inner cavity groups when Chl *a* is present in solution. The reduced tendency of the TM-β-CD to hydrogen bond interactions can also explain the 1:1 stoichiometry of its complexes with Chl *a* and the lower presence of the pigment aggregate form in these solutions.

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