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CHLOROPHYLLIDE *a* /CYCLODEXTRIN INTERACTION IN AQUEOUS SOLUTION

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Abstract

The interactions between Chlorophyllide *a* (Chlide), a pigment having the same structure of Chl *a* without the phytilic tail, and two CDs having the same moieties but different cavity size, the hydroxypropyl-β-cyclodextrin and the hydroxypropyl-γ-cyclodextrin, were studied in aqueous solutions by means of absorption, fluorescence spectroscopy, circular dichroism and isothermal titration calorimetry. The results obtained indicate that both cyclodextrins are able to modify the aggregation equilibrium of the pigment favouring the monomeric form.

Keywords: Chlide, hydroxypropyl-β-cyclodextrin, hydroxyl-γ-cyclodextrin, photodynamic therapy

Introduction

Recently a systematic study on the use of natural porphyrins combined with cyclodextrins (CDs) as potential sensitizers for the photodynamic destruction of tumor cells was undertaken in our laboratory [1–3]. The use of the natural porphyrins as sensitizers in photodynamic cancer therapy is a subject of interest because these compounds, having extended π electron systems, are usually characterised by intense absorption bands in the 600–850 nm wavelength region, where light has the maximal depth penetration into mammalian tissues [4, 5]. In addition to this characteristic and to some photophysical properties such as photostability and long lifetime of the photoexcited triplet state, a sensitizer to be effective for photodynamic therapy must also have a higher affinity for the target tissue than for the other tissues [6, 7]. Although it is difficult to predict if a compound has this selectivity, it is generally accepted that the selectivity is enhanced when the photosensitizer is amphiphilic or possesses a high lipophilic character. Unfortunately in the case of porphyrins, the increasing of the lipophilic character fosters the tendency of the molecules to aggregate with a consequent inhibition of the photosensitizing activity. The preliminary results of the spectroscopic study on aqueous solutions of chlorophyll a (Chl *a*) and cyclodextrins indicate that CDs are suitable carriers of Chl *a* in water. In fact, the CD

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presence in solution increases the Chl *a* solubility in water and makes it possible to shift the equilibrium aggregate-monomer towards the monomer. In particular, among the various CDs taken into exam, the β-CDs resulted to be the most effective. In aqueous solution of α-CD and HP-γ-CD 0.1 M, in fact, the pigment ($1 \cdot 10^{-5}$ M) is present to a large extent in the form of aggregates while in solution of β-CDs the absorbance of the monomeric form is comparable (HP-β-CD) or preponderant (DIMEB) to the absorbance of the aggregated form [2]. The different behaviour of Chl *a* in aqueous solution of hydroxypropyl-β-cyclodextrin and in aqueous solution of methylated-β-CD is also clearer in salt solution [3] where the pigment is mostly aggregated. Further evidence of the different ability of the two β-CDs to control the pigment aggregation is given by the intensity of the fluorescence of Chl *a* in HP-β-CD solutions which is lower than that obtained in DIMEB solutions at the same concentration [2].

The interactions between Chl *a* and β-CDs in aqueous solution have also been studied by calorimetric measurements [1]. Although the very low solubility of Chl *a* in water along with its high tendency to aggregate made it impossible to obtain quantitative thermodynamic information on the binding process between Chl *a* and β-CDs, calorimetric data indicated that Chl *a* interacts with CDs mainly as monomer and that the Chl *a* binds DIMEB more strongly than HP-β-CD producing exothermic and endothermic effects, respectively.

Spectroscopic and calorimetric data collected up to now do not provide clear clues on the action mechanism of CDs both in favouring the solubilization of the pigment and in promoting the conversion of its aggregated forms in the monomer. In order to study this mechanism it is essential to understand the role played by the long hydrophobic tail of Chl *a* in the interaction of the pigment with CDs. For this reason we have extended our study to the case of Chlorophyllide *a* (Chlide), a pigment which has the same structure of Chl *a* but lacks the phytilic tail [8]. In particular we performed a spectrophotometric and calorimetric study of the interaction between Chlide and two CDs having the same moieties but different cavity size: the HP-β-CD and the HP-γ-CD.

Materials and methods

Hydroxypropyl-β-cyclodextrin (HP-β-CD) *DS*=5.6 and hydroxypropyl-γ-cyclodextrin (HP-γ-CD) *DS*=4.8 were purchased form Aldrich and used without further purification. Chlide was synthesized and purified according to Fiedor *et al.* [9].

Visible absorption spectra were recorded using a Varian CARY/3 spectrophotometer. Fluorescence measurements were carried out using a Varian Cary Eclipse fluorescence spectrometer exciting at 420 nm. The circular dichroism spectra were recorded using a Jasco J810 spectropolarimeter. Calorimetric measurements were performed using a LKB 2277 (TAM) microcalorimeter equipped with a Thermometric 2250 titration unit. The isothermal titration calorimeter (ITC) was calibrated electrically and its performance was tested as previously described [10]. Binding experiments were carried out by injection 20 μL aliquots of Chlide aqueous solutions at dif-

ferent concentrations into the sample cell containing 1 mL of a CD solution (1 mM). Dilution experiments were carried out replicating the corresponding binding experiments but filling the sample cell or the syringe with water. The dilution experiments of CD solutions did not give rise to detectable thermal effects within the sensibility of the calorimeter in agreement with previous experiments [10]. It was not possible to perform inverse binding experiments, filling the sample cell with Chlide solutions, because the thermal equilibration of the sample cell requires a long time during which part of Chlide can become pheophorbide.

The solutions of Chlide were prepared sampling the appropriate amount of an alcoholic stock solution of Chlide, evaporating it with a stream of dry nitrogen and redissolving the obtained thin film of pigment in the proper quantity of water or of CD solution. The residuals of Chlide not dissolved were recovered with ethanol, quantified by absorbance measurements and used to correct the solution concentrations.

Results and discussion

In Fig. 1 the electronic absorption spectra of Chlide are shown at different concentration in the range from $4 \cdot 10^{-6}$ to $5 \cdot 10^{-5}$ M. The spectra are characterized by an intense Soret band at 420 nm and a Qy (0,0) band in the red region at 668.7 nm and do not present significant differences both in the shape and position of the absorption bands with the increase of the pigment concentration. Due to the complicated multicomponent structure of the Soret band, the interest has been focused on the Chlide Q_y transition, which is indicative of the presence of aggregated species in solution [11]. Enlargement and shifts of this band are in fact associated with the presence of pigment dimer or larger aggregates [12–14]. The FWHH (35 nm) of the red band of the samples reported in Fig. 1 indicates that part of the pigment is present in oligomeric form in agreement with what is reported in [12]. The presence of pigment aggregates in the investigated range of concentration is also confirmed by the CD spectra reported in Fig. 2. The presence of a non conservative red band splitting characterized by a positive band at 703 nm and a negative one at 684 nm is evident. This splitting together with the weaker one in the Soret region have already been attributed to the presence of an aggregated form of Chlide in surfactant aqueous system [15].

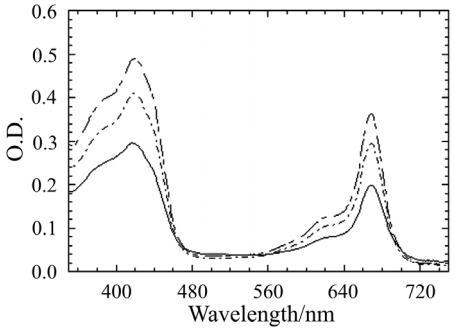

Fig. 1 Absorption spectra of Chlide (—) – $4 \cdot 10^{-6}$ M, (–·–) – $7 \cdot 10^{-6}$ M, and (– - –) – $1 \cdot 10^{-5}$ M

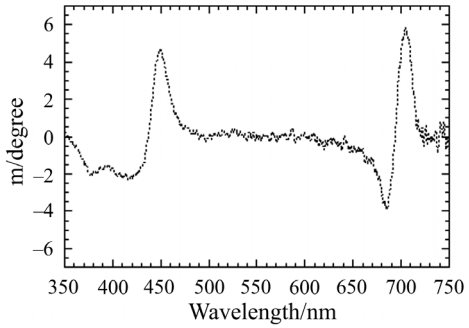

Fig. 2 Circular dichroism spectra of a $1 \cdot 10^{-5}$ M Chlide solution in water

Figure 3 shows the absorbance spectra of Chlide $1 \cdot 10^{-5}$ M in aqueous solution of HP-β-CD (Fig. 3a) and HP-γ-CD (Fig. 3a) at different CD concentrations. In both cases, the presence of CD gives rise to an increase of the Chlide absorbance and a narrowing of the Q_y band to about 25 nm. This effect is more evident in HP-γ-CD solution where the maximum value of the absorbance is already obtained at the lowest CD concentration used (0.05 M), as shown in the inset of the same figure. On the contrary in the HP-β-CD solutions the Chlide peak intensity gradually increases when the CD concentration is increased. Moreover the Chlide spectra in CD solutions evidence the vibronic peaks associated to both the Q_y and Q_x bands which are not resolved in the spectrum of the pigment in water as shown by a deconvolution analysis of the spectra not reported. This behaviour can be ascribed to the conversion of the aggregated form of the pigment into the monomer promoted by the interaction with the CDs and to the chromophore transfer from the polar aqueous environment to the apolar cavity of the CDs.

Circular dichroism spectra (Fig. 4) confirm the presence of the pigment monomeric form in CD solutions. Both spectra show the presence of non-conservative peaks of low intensity at 668 and 374 nm characteristic of the monomeric form.

The presence of an interaction between the Chlide and CDs is supported also by the increase of the fluorescence intensity increasing the CD concentration (Fig. 5). In fact it is well known that chlorophyll aggregates do not show any detectable fluorescence because of an internal energy transfer between the porphyrinic rings [16]. In general the chromophore inclusion in CD cavity provokes an increase of the fluorescence signal which is caused by a decrease of intramolecular rotational freedom of the chromophore molecules in the restricted microenvironment and by its protection from quenching effects. The slight difference in the Chlide fluorescence intensity obtained in the two CD solutions can be the result of the different cavity size. The larger cavity of the HP-γ-CD, even if it favours the pigment inclusion, offers a less restricted microenvironment compared to the HP-β-CD, and consecutively to less intensive fluorescence signals as shown in Fig. 5.

As already observed in the calorimetric study of the interactions between Chl *a* and CD [1], also in the case of Chlide *a* detectable thermal effect was obtained only

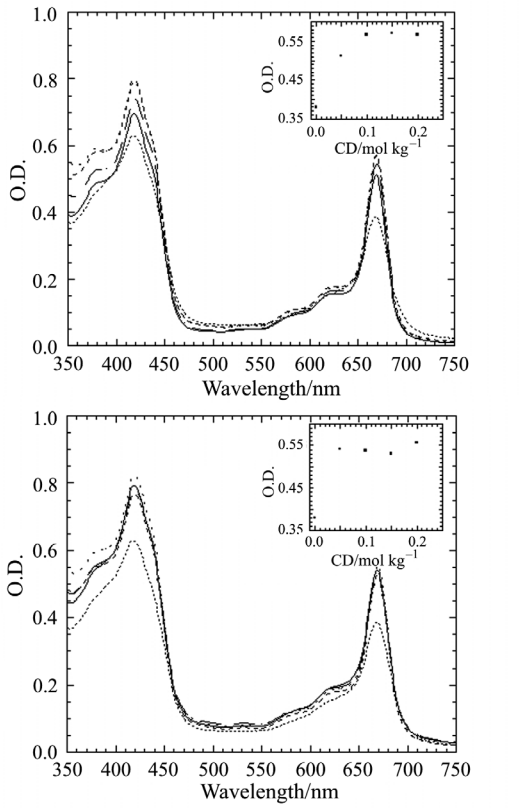

Fig. 3 Absorption spectra of Chlide $1 \cdot 10^{-5}$ M in aqueous solution of a – HP-β-CD and b – HP-γ-CD at different CD concentrations: (·····) – 0 M, (—) – 0.05 M, (–·–·) – 0.10 M, (– – –) – 0.15 M and(----) – 0.2 M. The corresponding absorbance of Chlide at 668.7 nm as a function of the CD concentration are re-

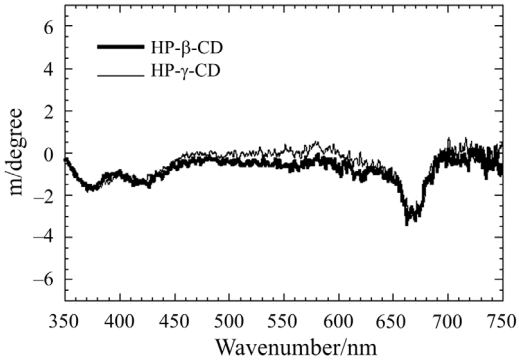

Fig. 4 Circular dichroism spectra of Chlide $1 \cdot 10^{-5}$ M in aqueous of HP-β-CD (0.2 M) and of HP-γ-CD (0.2 M)

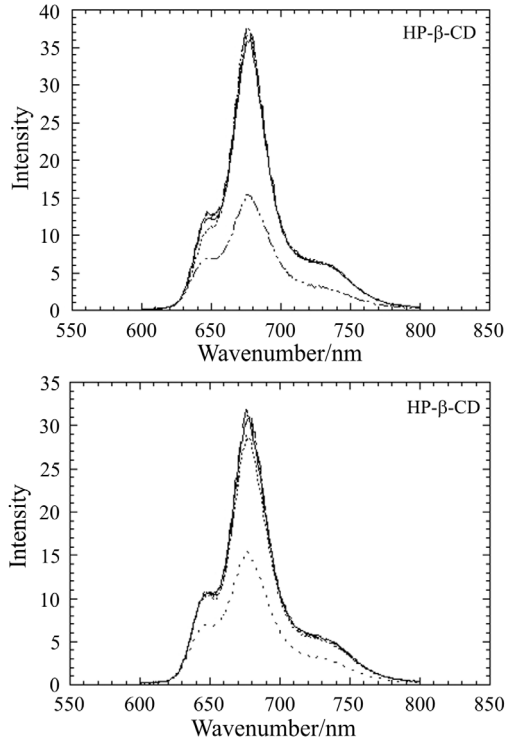

Fig. 5 Fluorescence spectra of Chlide $1 \cdot 10^{-5}$ M in aqueous solution of a – HP-β-CD and b – HP-γ-CD at different CD concentrations: (–···–) – 0 M, (----) – 0.05 M, (–·–) – 0.10 M, (– – –) – 0.15 M and (·····) – 0.2 M

using CD concentrations much higher than those required to achieve a complete titration. Consequently it was not possible to analyse the dependence of recorded heats on the Chlide/CD ratio as suggested in literature and to obtain thermodynamic information such as the heat of binding, the association constant, the change in entropy and the stoichiometry of the complex [17, 18].

Figure 6 shows, as example, a section of two experimental power–time plots obtained by the calorimetric study. The plot reported in the Fig. 6a refers to the dilution experiment of a Chlide solution, whereas that reported in the Fig. 6b refers to the binding experiment of Chlide with HP-β-CD. The power–time plots associated to the binding experiments of Chlide with HP-γ-CD are very similar to those obtained with HP-β-CD and therefore are not shown. At the concentration used in these experiments, $7 \cdot 10^{-5}$ M, Chlide has the same spectroscopic characteristics of the more diluted solutions used in the spectroscopic study and therefore the pigment is partly aggregated. The thermal peaks associated to successive injections of a Chlide solution to a CD solution are broader than those associated to the dilution of Chlide solution. This difference clearly indicates that a thermal effect is associated to the interaction between Chlide and HP-CD; this effect is endothermic as already observed in the case of Chl *a* but differs in

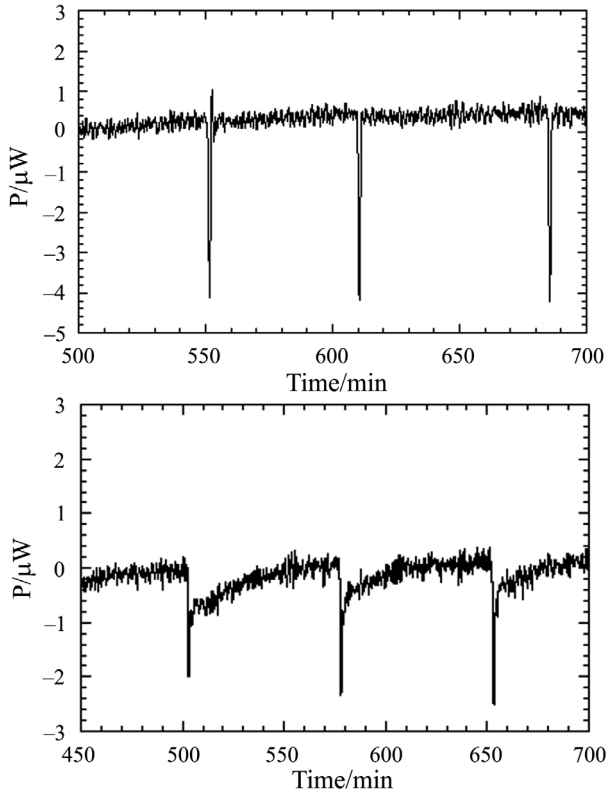

Fig. 6 Experimental power–time plot associated to successive injections of an aqueous Chlide a – $7 \cdot 10^{-5}$ M solution in water and b – in a 1 mM HP-β-CD solution

the time evolution. After each injection, in fact, the calorimetric signal has difficulties in returning to the baseline value in spite of the long delay between two successive injections. This behaviour suggest that the mixing of the Chlide solution with the CD solution produces slow thermal processes. In order to understand the reasons for such behaviour, the calorimetric study was also performed using a more diluted solution of Chlide ($1 \cdot 10^{-5}$ M). The experimental power–time plot obtained at such a concentration is characterised by sharp peaks, very similar, also if more intense, to those obtained during the dilution experiments of Chlide or studying the interaction between Chl *a* and CD. Therefore at a low Chlide concentration the thermal processes associated to the mixing of a Chlide solution with a CD solution are faster than those obtained at a higher concentration. Since the main difference between the two solutions is the relative amount of pigment in the aggregate state, it is possible to infer that the CD interacts with the pigment mainly as monomer, as already observed for the Chl *a*. The slow thermal processes observed in the case of high Chlide concentration can be due the disaggregation processes of the pigment which makes more effective the interaction between the pigment and the CDs possible.

Conclusions

In conclusion, the overall spectroscopic and calorimetric data presented in this study indicate that both β and γ hydroxypropyl-CD favour the presence in aqueous solution of Chlide as monomer differently from what was observed in the case of Chl *a*. This result indicates that the interactions between the CDs and the porphyrinic ring have an important role in the CD's ability to modulate the aggregation properties of porphyrins and that the differences in the behaviour of the two pigments are likely produced by their different tendency to aggregate.

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