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INTERACTION BETWEEN CHLOROPHYLL *a* AND β-CYCLODEXTRIN DERIVATIVES IN AQUEOUS SOLUTIONS
Spectroscopic and calorimetric study

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Abstract

Cyclodextrins (CDs) are widely used as delivery systems of poorly water soluble drugs in pharmacological applications. The delivery system produces an increase of aqueous solubility of the drug and significant modifications in its physico-chemical and pharmacological properties.

In this paper we report the results of our study on aqueous solutions of chlorophyll *a*, a natural pigment useful as sensitizer in the photodynamic therapy, and two CDs: hydroxypropyl-β-cyclodextrin and heptakis(2,6-di-O-methyl)-β-cyclodextrin.

The interactions between chlorophyll and CDs and the effect produced by the presence of the CDs on the aggregation of chlorophyll were studied by calorimetric and UV-Vis spectrophotometric measurements respectively.

Keywords: chlorophyll *a*, cyclodextrins, photodynamic therapy

Introduction

Cyclodextrins (CDs) are cyclic oligomers of glucose characterised by a very low toxicity and by the ability to form inclusion complexes with many organic substances, widely used as delivery systems of poorly water soluble drugs in pharmacological applications [1]. The complexation of a drug produces an increase of aqueous solubility of the drug and significant modifications in its physico-chemical and pharmacological properties. Consequently, an appropriate choice of the delivery system can yield to an improvement of the pharmacological efficacy of the drug.

Studies on the photodynamic therapy (PDT), a treatment of tumours which combine the use of a photosensitizer, endogenous oxygen and light [2], indicate that the

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use of CDs to delivery photosensitizers, such as porphyrins and analogs, is helpful to cope problems which limit the use of this treatment [3].

In general, a photosensitizer to be effective in PDT should have the following properties: a high chemical purity, a high molar extinction coefficient at the absorption maximum in the red spectral region, a low tendency to aggregate in aqueous media, a high lipophilic or amphipatic character, a good photostability, a long triplet lifetime and a high yield of $^1O_2$ [4]. Unfortunately, none of the compounds studied as photosensitizers has all these properties and sometimes structural modifications aimed at the fulfilment of some properties produces a worsening of other ones. For example, it was observed, in the case of porphyrins, that an increase of their lipophilic character produces a higher tendency to aggregate [5]. The use of CDs as delivery systems of porphyrins can be useful to control their aggregation and shift the equilibrium aggregate–monomer towards the monomer, hence increasing the photosensiting activity. Recently we have undertaken the study of aqueous solutions of a natural amphipatic porphyrin, Chlorophyll *a* (Chl *a*), which is the main pigment of green plants, in presence of two homologous CDs: hydroxypropyl-β-cyclodextrin (HP-BCD) and heptakis(2,6-di-O-methyl)-β-cyclodextrin (DIMEB).

In this study β-cyclodextrin derivatives were used because of the ability of dimers of β-CDs to form inclusion complexes with porphyrins, as already reported in literature [6]. In particular HP-BCD and DIMEB are two of the most frequently used CD derivatives in drug formulations and are generally preferred to natural CDs because of their higher aqueous solubility [7].

At this first stage of the work we studied the interactions between Chl *a* and β-CDs in aqueous solution by calorimetric measurements and the effect produced by the presence of CDs on the aggregation of Chl *a* using UV-Vis spectrophotometric measurements. The very low solubility of Chl *a* in water along with its high tendency to aggregate, made impossible to obtain quantitative thermodynamic information of the binding process between Chl *a* and β-CDs. Notwithstanding this limitation, calorimetric and spectrophotometric data have provided interesting information on the studied systems.

Materials and methods

Chlorophyll *a* (Chl *a*) was isolated from *Spirulina geitleri* [8]. Stock solutions in *n*-pentane were stored in the dark at –80°C under a N_2 atmosphere. Ether solutions of Chl *a* were used to determine spectrophotometrically the purity and the concentration of the samples [9]. Hydroxypropyl-β-cyclodextrin (HP-BCD) and heptakis-(2,6-di-O-methyl)-β-cyclodextrin (DIMEB) were purchased from Aldrich and used without further purification.

Appropriate amounts of freshly prepared stock solution were evaporated to dryness under a flow of N_2 gas. Then water or CD aqueous solutions were used to dissolve dry chlorophyll. All aqueous solutions were briefly sonicated for 5–10 min.

Visible absorption spectra were recorded using a Varian CARY/3 spectrophotometer. Calorimetric measurements were performed using an 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 15 μL aliquots of a Chl *a* aqueous solution (1 μM) into the sample cell containing 1 mL of a CD solution (10 mM). Dilution experiments were carried out replicating the corresponding binding experiment but filling the sample cell with 1 mL of water.

Results and discussion

Contrary to other amphipatic molecules, such as surfactants, Chl *a* is scarcely soluble in water also in form of aggregates [11]. Figure 1 shows the absorption spectrum of Chl *a* in aqueous solution. The spectrum evidences the presence of the monomeric form of Chl *a*, characterized by a maximum in the red region at 670 nm, according to previously reported data [12, 13], together with a long-wavelength form of the pigment with a maximum at 745 nm, indicative of Chl *a* aggregate formation [14]. The structure of this aggregate form has been described as planar sheet-like, in which two molecules of water are involved in the bridge connecting two Chl *a* molecules [15, 16]. In addition, although it was observed that, at increasing of Chl *a* concentration, the peak corresponding to the aggregate becomes more intense, it was not possible to correlate the intensity of the absorption bands of the monomeric and aggregated form of Chl *a* to their relative quantities. The enlargement of the band at 745 nm indicates the presence not of a single and well defined aggregate but of a non-uniform distribution of different Chl *a* aggregates. Therefore the intensity and shape of the absorption band at 745 nm is the convolution of spectra corresponding to each aggregate. In addition the Chl *a* water solubilization process is kinetically controlled, hence it is difficult to prepare Chl *a* aqueous solutions with the same relative amounts of monomer and aggregated forms.

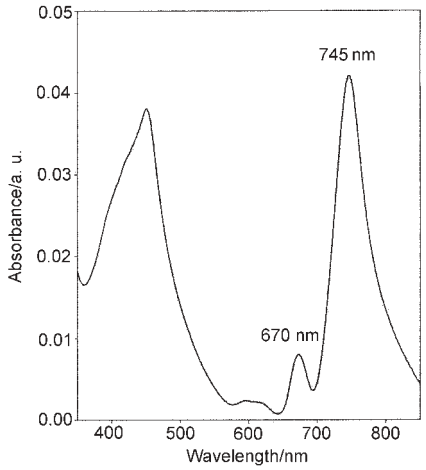

Fig. 1 Absorption spectrum of a Chl *a* aqueous solution

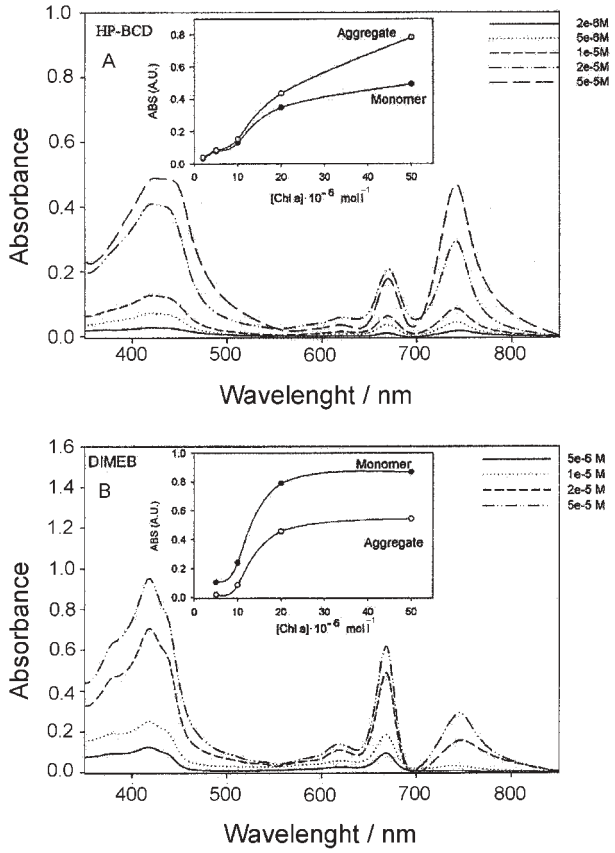

Fig. 2 Absorption spectra of Chl *a* in solution of A – HP-BCD 0.5 M and B – DIMEB 0.5 M at increasing Chl *a* concentration. The corresponding absorbance of Chl *a* at 670 and at 745 nm associated to the monomeric and aggregated species respectively are reported in the insets

Figure 2 shows the absorbance spectra of Chl *a* in solution of HP-BCD and DIMEB ([*CD*]=0.5 M) at increasing Chl *a* concentration. The Chl *a* concentrations of these solutions are higher than those obtained in water since the pigment was directly dissolved in CD aqueous solutions which increase Chl *a* solubility.

The spectra indicate that in solution of HP-BCD Chl *a* (Fig. 2A) is prevalently present as aggregated form whereas in solution of DIMEB (Fig. 2B) the monomeric form is preponderant. In particular (inset Fig. 2A) in HP-BCD the ratio between the absorbance relative to the Chl *a* aggregate and monomer is pratically the same at pigment concentration lower than 10^{-5} M, while increases with increasing Chl *a* concentration. In DIMEB, instead, the prevalence of the absorbance relative to the monomeric Chl *a* form, was observed at all pigment concentrations studied. It is interesting to note (inset Fig. 2B) that the ratio between monomer and aggregate form increases at increasing the Chl *a* concen-

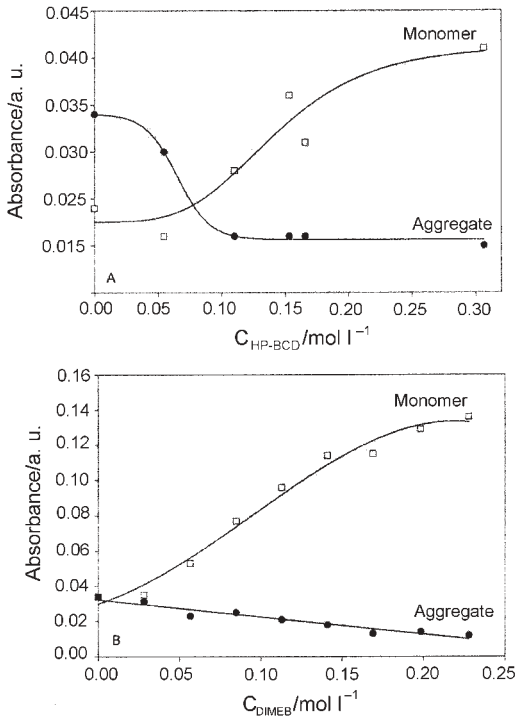

Fig. 3 Chl *a* absorbance of aggregate and monomer respectively are reported as a function of CD concentration where different concentrations were obtained adding increasing amounts of CD to a $1 \cdot 10^{-6}$ M Chl *a* aqueous solution

tration up to a pigment concentration of $2 \cdot 10^{-5}$ M. At higher Chl *a* concentration no changes of this ratio were observed. The data obtained evidence that it is possible to modulate the presence of aggregated and monomeric forms of Chl *a* in solution by varying the nature of CD, the ratio between pigment and CD concentrations and the sample preparation methods. The last dependence is confirmed by Fig. 3, where the Chl *a* absorbance of aggregate and monomer respectively, reported as function of CD concentration, were obtained adding increasing amounts of CD to a $1 \cdot 10^{-6}$ M Chl *a* aqueous solution. In this case we observed that the aggregated form is prevalent up to a HP-BCD concentration of $1.1 \cdot 10^{-2}$ M, while a reversal of trend is obtained at higher CD concentrations (Fig. 3A). For DIMEB, instead, it is again observed the prevalence of monomeric Chl *a* form at all CD concentrations studied. Comparing these results with those reported in Fig. 2 it is possible to point out that the sample preparation method plays an important role in stabilizing one Chl *a* form respect to the other one mainly for HP-BCD.

Calorimetric experiments were performed using solutions of Chl *a* the concentration of which is at the limit of chlorophyll solubility in water since the use of more diluted solutions did not produce any detectable signal. At these concentrations Chl *a* is present partly as monomer and partly as aggregate. Furthermore it is important to observe that ca-

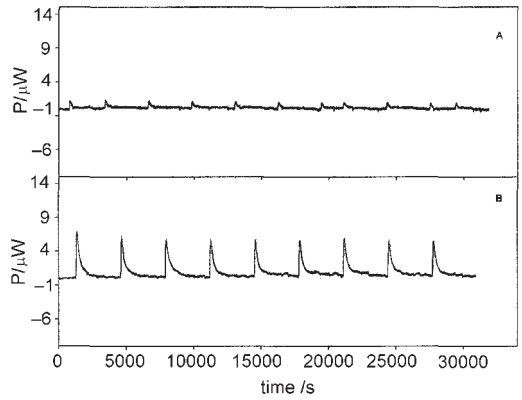

Fig. 4 Experimental power-time plot associated to successive injections of an aqueous Chl *a* solution where Chl *a* is largely present in solution as aggregate A – and as monomer B – in a 10 mM hepta-β-CD solution

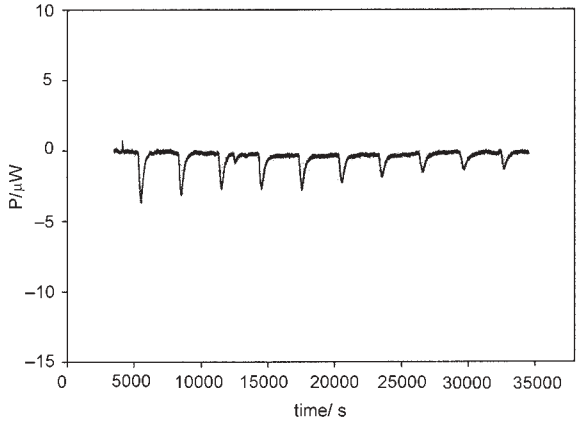

Fig. 5 Experimental power-time plot associated to successive injections of an aqueous Chl *a* solution where Chl *a* is largely present in solution as monomer in a 10 mM β-CD solution

lorimetric experiments were carried out using Chl *a* solutions, the concentration of which is about 10^{-5} times lower than that of CD and so the curves reported in this study refer to partial titrations. Consequently, it was not possible to analyse the dependence of recorded heat on the Chl *a*/CD ratio as suggested in literature [17] and obtain thermodynamic information such as the heat of binding, the association constant, the change in entropy and the stoichiometry of the complex. From a qualitative point of view it was observed that the results obtained depend on the aggregation state that Chl *a* has in the aqueous solution used to titrate the CD solutions. If Chl *a* is largely present in the solution as aggregate the titration produces a very low thermal effect as shown in Fig. 4A. On the contrary, if Chl *a*

in the solution is mainly monomer, the titration gives rise to a detectable thermal effect (Fig. 4B). The dilution experiments of Chl *a* solution in water did not give rise to detectable thermal effects within the sensibility of the calorimeter used. The same result was obtained from the dilution experiments of CDs solutions in agreement with previous experiments [10]. Therefore, the thermal effects detected in the binding experiment have to be ascribed to the binding of Chl *a* with CDs. These findings suggests that the monomer Chl *a* is mainly involved in the interaction with CDs.

The interaction of Chl *a* with HP-BCD produces an endothermic effect (Fig. 5), whereas the interaction with DIMEB produces an exothermic effect (Fig. 4). The different observed behaviour between DIMEB and HP-BCD indicates an opposite dependence of their binding equilibrium with Chl *a* on the temperature and suggests that Chl *a* interacts differently with the two CDs. Considering that the interactions responsible for the association of guest molecules with β–CD are mainly van der Waals interactions, hydrogen bonds and hydrophobic interactions, it is likely that this last one has a relevant role in the interaction between HP-BCD and Chl *a* since this type of interaction gives rise to a positive change in the entropy which, overwhelming the unfavourable change in the enthalpy, makes the binding process spontaneous.

Curves reported in Fig. 4 show that successive injections of a Chl *a* solution in DIMEB solutions produce almost constant thermal effects. Instead, in the case of HP-BCD (Fig. 5) successive injections of the same Chl *a* solution produce decreasing thermal effects. Since both curves refer to uncomplete titrations the difference between them suggests that the binding constant of Chl *a* with DIMEB is higher than that with HP-BCD.

The presence of strong interactions between Chl *a* and DIMEB was also deduced by circular dichroism measurements and by the higher stability of Chl *a* toward photooxidation in aqueous solutions of DIMEB than in those of HP-BCD [18].

At the moment in our laboratory new procedures of Chl *a* solubilization in water, aimed at obtaining more concentrated pigment aqueous solutions, are object of study. Availability of these solution will allow us to continue the study obtaining quantitative information on the Chl *a*/CDs inclusion complexes such as the binding constant and the ratio host/guest and so to check our hypothesis.

Conclusions

The results of this study indicate that β-CDs are suitable carriers of Chl *a* in water, increasing Chl *a* solubility in aqueous solutions and making possible the modulation of aggregated and monomeric forms of Chl *a* in solution by varying the nature of CD, the ratio between pigment and CD concentrations and the sample preparation method.

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