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Photophysical and electrochemical properties of chlorophyll *a*–cyclodextrins complexes

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Dedicated to Prof. Mario Della Monica (January 9th, 1932–September 9th, 2003)

Abstract

In this work, we have studied the interactions between two different cyclodextrins (CDs) and chlorophyll *a* (Chl *a*) in the presence of electrolyte by means of absorption, fluorescence spectroscopy, circular dichroism and cyclic voltammetry. The results obtained indicate that the presence of both CDs gives rise to an increase of Chl *a* solubility in water. In particular, heptakis-(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TRIMEB) favours the dissolution of Chl *a* monomer in aqueous solution, whereas the presence of hydroxypropyl-β-cyclodextrin (β-HP-CD) promotes the pigment aggregation.
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1. Introduction

Photodynamic therapy is an attractive modality to treat cancer and hyperproliferative diseases based on the use of a photosensitizer in the presence of oxygen and light [1]. The effectiveness of PDT treatment largely depends on photochemical and photophysical properties of the sensitizer and on biochemical composition and characteristics of the target tissue [2]. Among the various classes of explored compounds as potential photosensitizers in PDT, porphyrins and their analogs are considered the most promising since their spectra are usually characterized by intense absorption bands in the 600–850-nm wavelength region, where the maximal light depth penetration into tissues is obtained [3]. It is generally accepted that tumour selectivity is enhanced with the increasing of the lipophilic character of the photosensitizer or when it evidences amphiphilic properties. As a consequence, its pre-incorporation into suitable delivery systems is required. Cyclodextrins (CDs) are cyclic oligomers of glucose characterised by a very low toxicity and by the ability to form inclusion complexes with many or-

ganic substances, widely used as delivery systems of poorly water-soluble drugs in pharmacological application [4]. Consequently, 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, which is the photosensitive specie [5]. Among natural amphipathic porphyrin, chlorophyll *a* (Chl *a*), which is the main pigment of green plants, has been receiving interest as photosensitizer. Chl *a* is virtually insoluble in water, like almost all other naturally occurring chlorophylls having a long chain esterifying alcohol. In fact, the very small amount of Chl *a*, which is possible to solubilize in pure water, is present to a large extent as polymer [6]. Preliminary results, obtained in our laboratory, have demonstrated that the addition of CD to the Chl *a* increases pigment solubility in aqueous solutions and makes possible the modulation of aggregated and monomeric forms of Chl *a* in solution by varying the nature of CD, the ratio between the pigment and CD concentration and the sample preparation method [5].

In order to study the electrochemical properties of complexes Chl *a*/CD, we have extended the investigation to the case of aqueous solutions containing electrolyte (lithium perchlorate). The study has been carried out at different pigment and CD concentrations using two homologous

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CDs, hydroxypropyl-β-cyclodextrin (β-HP-CD) and heptakis-(2,3,6-tri-O-methyl)-β-cyclodextrin (TRIMEB), differing in the functionalization of the hydroxyl groups.

2. Experimental

2.1. Materials and methods

Chlorophyll *a* was isolated from spinach leaves [7]. Ether solutions of Chl *a* were used to determine spectrophotometrically the purity and the concentration of the samples [8]. The concentration of the pigment was determined by its absorbance in the red band peak using the molar extinction coefficient in the same solvent [9]. Hydroxypropyl-β-cyclodextrin, DS = 5.6, heptakis-(2,3,6-tri-O-methyl)-β-cyclodextrin, lithium perchlorate and all the solvents used were purchased from Aldrich and used without further purification. With regard to the sample preparations, the appropriate amounts of alcoholic stock solution of Chl *a* and cyclodextrin were mixed together and evaporated with a stream of dry nitrogen. The thin films of pigment and CD were redissolved in the desired quantity of 0.1 M salt solution. Visible absorption spectra were recorded using a Varian CARY/3 spectrophotometer. Fluorescence measurements were carried out using a Varian Cary Eclipse fluorescence spectrophotometer exciting at the maximum absorption of Chl *a* in the Soret band. The circular dichroism spectra were recorded using a JASCO J810 spectropolarimeter. Electrochemical experiments were performed in a three-electrode cells using as working electrode a mercury film electrode (MFE). Voltammograms were recorded using the AUTOLAB PGSTAT10 potentiostat interfaced with a personal computer.

3. Results and discussion

The absorption spectra of Chl *a* in TRIMEB solutions at different CD concentration are shown in Fig. 1. The spectra are characterized by an intense Soret band at 435 nm and a Q_y (0,0) band around 672 nm. The batocromic shift of about 5 nm for the Soret band and $10-12$ nm for the transition Q_y (0,0), with respect to absorption spectra characteristic of the Chl *a* monomeric form in ethyl ether, can be ascribed to the presence of both monomer and hydrated dimeric forms of Chl *a* in solution as previously reported for Chl *a* in solution of surfactant and water/organic solvent mixture, although at Chl *a* peak at 670 nm it has been assigned to the monomeric form of the pigment in heptakis-(2,6-di-O-methyl)-β-cyclodextrin and β-HP-CD solutions in the absence of electrolyte [6]. The shift from 672 to 668 nm of the peak in the red region observed in increasing the cyclodextrin concentration suggests an increased interaction between pigment and CD with rising CD concentration. This hypothesis is confirmed by fluorescence spectra of Chl *a* at different concentrations
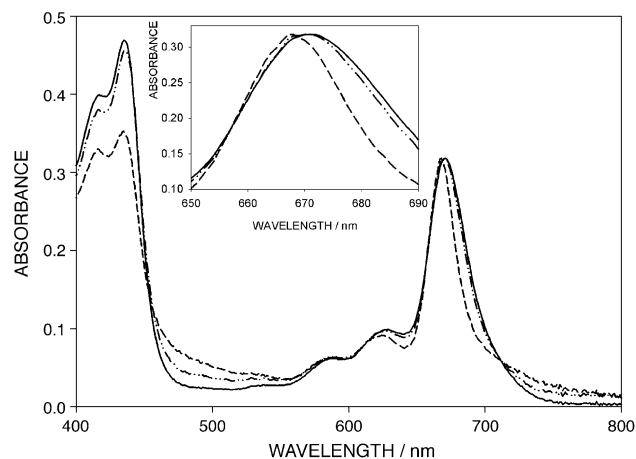

Fig. 1. Absorption spectra of Chl *a* (5×10^{-5} M) in different concentration of TRIMEB: (—) 0.05 M, (—·—) 0.1 M, (---) 0.15 M. Inset: enlargement of the peak in the red region of the spectra.

of TRIMEB reported in Fig. 2A, where a gradual increase of the fluorescence signals is observed with increasing the CD concentration in solution. These results can be explained assuming an increased pigment interaction between Chl *a* and CD, which would cause a decrease of intramolecular rotational freedom of the pigment molecule in the restricted microenvironment and its protection from external quenchers such as electrolyte ions [10]. Moreover, since chlorophyll aggregates are typically not fluorescent species because of the dipole–dipole energy transfer between the porphyrinic rings [11], the fluorescence signals recorded in TRIMEB solution suggest that the observed absorption peak at 672 nm can be attributed to the monomeric form of the pigment.

Circular dichroism spectrum of aqueous solutions of Chl *a* in TRIMEB (Fig. 2B) confirms the presence of only monomer Chl *a* in solution. In fact, it is possible to observe the presence of non-conservative peaks of low intensity at 672 and 450 nm, very similar to the peaks that characterize the circular dichroism spectra of Chl *a* monomer in ether.

Conversely to TRIMEB solution, Chl *a* shows a different behaviour in β-HP-CD solutions. Absorption spectra recorded at different time from sample preparation (Fig. 3) show, in fact, in addition to the peak at 672 nm, the presence of a peak at 740 nm. The last mentioned absorption band is a characteristic of the aggregated form of the pigment whose structure has been described both as planar sheet-like in which two molecules of water are involved in the bridge connecting two pigment molecules, [12] and as cylindrical micelle [13]. The amount of aggregated form rises increasing the CD concentration (insert, Fig. 3). This behaviour can be ascribed to the increased ionic strength and dielectric constant of solution as previously reported for Chl *a* in microheterogeneous systems of ionic surfactant [14] and in water-rich region of water–acetonitrile mixture [15]. In general, the presence of electrolytes in solution can affect the interaction between two molecules and therefore their
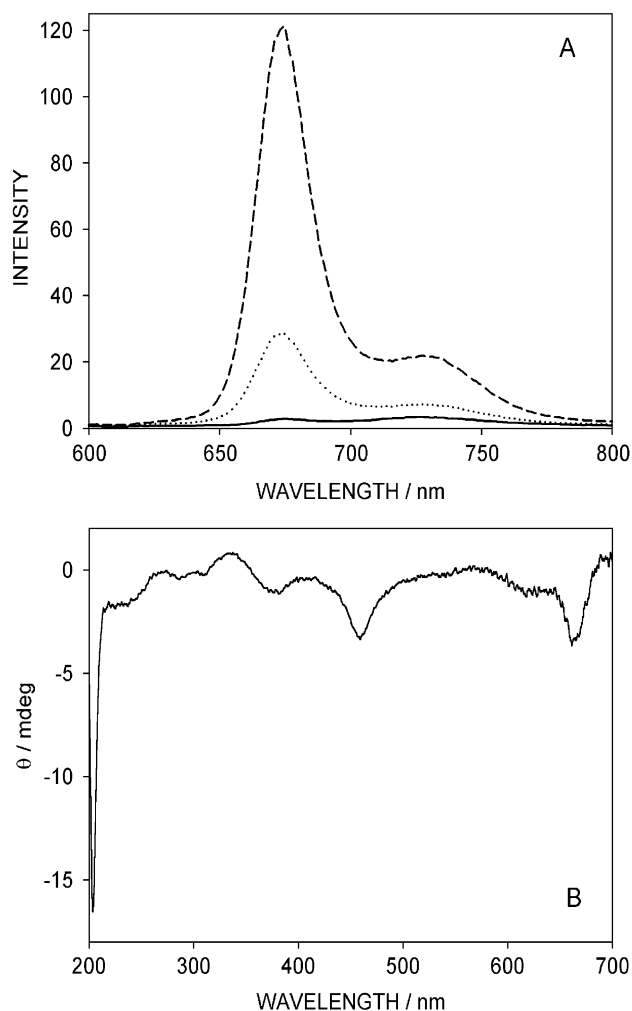
Fig. 2. (A) Fluorescence spectra of Chl *a* $(5 \times 10^{-5}$ M) at different concentration of TRIMEB: (—) 0.05 M, ($\cdots$) 0.1 M, (---) 0.15 M. (B) Circular dichroism spectra of Chl *a* $(5 \times 10^{-5}$ M) in 0.05 M TRIMEB.

Fig. 3. Time evolution spectra of Chl *a* $(5 \times 10^{-5}$ M) in β-HP-CD 0.15 M: (—) 0 h, ($\cdots$)1 h, (---) 2 h, (—$\cdot$—) 3 h, (– – –) 4 h. Inset: dependence of the absorbance at 740 nm on the β-HP-CD concentration.

ability to bind by means of different mechanisms. Neglecting the effects due to a direct participation of ions, which are not important in the case of binding between neutral molecules, the ions in solution produce changes in ionic strength and in water activity [16].

Usually, an increase of the ionic strength produces the aggregation of the pigment [14] minimizing in this way the hydrophilic interactions. The TRIMEB presence in solution offering a more hydrophobic environment suitable for the pigment not constrained it to aggregate allowing us to obtain the Chl *a* monomeric form in solution and thus a system useful for photodynamic therapy. The β-HP-CD instead contributing only to the increasing of the ionic strength favours the pigment aggregation.

Since one of the requirement for an ideal photosensitizer is the low tendency to aggregate in aqueous media, our attention has been focused on the TRIMEB solution where the pigment is present in monomeric form.

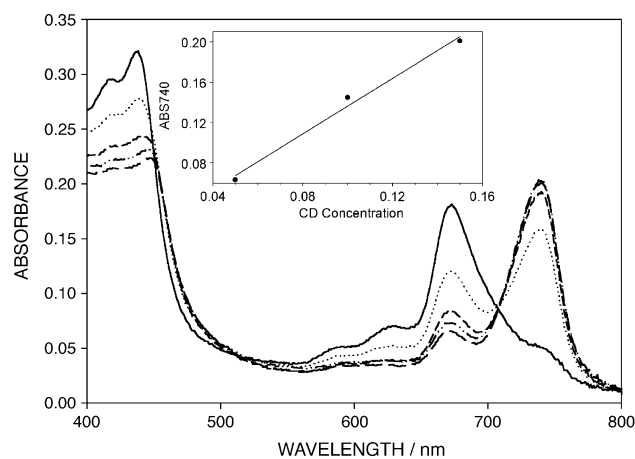The interaction between the pigment and TRIMEB has also been studied by a completely independent method, the

cyclic voltammetry. In Fig. 4 (continuous line), the voltammogram relative to 2×10^{-5} M Chl *a* in 0.05 M TRIMEB is shown. By comparison, the electrochemical response of the pigment in a 70/30 v/v acetone/water system is also reported (dashed line). In the binary mixture, the voltammogram of Chl *a* evidences two reduction peaks at -0.895 and -1.269 V (vs. NHE). As previously reported in literature, the two peaks, whose relative intensity is dependent on the water content in solution, are attributable to the irreversible reduction of porphyrin ring to its radical anion of two different solvated forms of monomeric Chl *a*; the first peak at more positive potential can be ascribed to the reduction of acetone solvated Chl *a*, while the second to the dihydrated form of the monomer [11,17–19].

In the voltammogram relative to the same Chl *a* concentration in TRIMEB solution (continuous line), three main reduction peaks at -0.541, -0.998 and -1.277 V (vs. NHE) appear. The low current intensity of the peaks relative to the formation of the radical anion can be put in relation
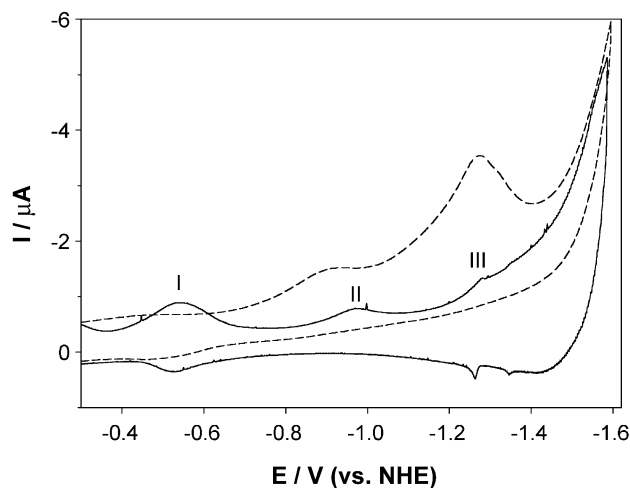

Fig. 4. Cyclic voltammograms of Chl *a* $(5 \times 10^{-5}$ M) in 0.05 M TRIMEB (—) and 70/30 v/v acetone/water (---) recorded at a scan rate of 200 mV/s.

with the shielding effect of CD that decreases the diffusion coefficient of the Chl a molecule and the exposition of the pigment to the electrode surface. Because the size of the cyclodextrin cavity does not allow the inclusion of the Chl a macrocycle, the shielding effect on the Chl a current intensity can be related alternatively to the involvement of two CDs in the inclusion complex or to a folding of the pigment, with exposure of the carbonyl group towards the solution. The second hypothesis seems to be supported by the appearing in the figure of a reduction peak (peak I), at the potential typical of the reduction of the C=O group on the V cyclopentanonic ring of the macrocycle [20]. The noticeable enhancement of the carbonylic reduction signal in the sample containing the CD indicates a structural organization of the Chl a–CD complex in which the C=O groups are more exposed favoring the preferential interaction of the molecule with the mercury surface throughout it [10,21,22]. Quite remarkably, the peak relative to the hydrate form of the pigment is not anymore present in the voltammogram, indicating the more hydrophobic environment around the porphyrin ring. It is also evident that there is a general decrease of the capacitive current indicating that, in this system, there is a greater coverage of the electrodic surface [22].

4. Conclusion

In conclusion, the overall data presented in this study indicate that the methylation of the OH groups in TRIMEB favors the interaction between CD and pigment probably because of an increased apolarity and CD cavity size variation. The presence of electrolyte further enhances this interaction.

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