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Phototoxicity and cytotoxicity of chlorophyll *a*/cyclodextrins complexes on Jurkat cells

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ABSTRACT

The aggregation status of chlorophyll *a* (Chl *a*) and the ability of four cyclodextrins, hydroxypropyl-β-cyclodextrin (HP-β-CD), hydroxypropyl-γ-cyclodextrin (HP-γ-CD), heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DIMEB), and heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TRIMEB), to solubilize the pigment in the complete cellular medium RPMI 1640 was estimated by means of UV–Vis absorption and static resonance light scattering (RLS) measurements. The results indicate that the pigment interacts with cyclodextrins in the cellular medium differently to that observed in water. The cytotoxic and phototoxic activity of these complexes towards human leukemia T-lymphocytes (Jurkat cells) was tested by means of experiments aimed to discriminate between the intrinsic toxicity and the toxicity induced by light.
The overall data indicate that the HP-β-CD is the cyclodextrins having the best characteristics to form with Chl *a* a potential supramolecular system for the photodynamic therapy.

1. Introduction

Photodynamic therapy (PDT) is a clinically approved treatment for several types of cancer and hyperproliferative diseases. PDT requires the simultaneous presence of three components, a sensitizer, light and molecular oxygen. The photochemical interaction of the sensitizer and light in presence of molecular oxygen produces reactive oxygen species (ROS) which damage some cellular components leading to the destruction of tumor cells by apoptosis or necrosis cell death [1].

In the last few years PDT has been subject of growing interest because this therapy has the advantage to have a good selectivity which makes its side effects much more tolerable than those associated to other treatments as for example chemotherapy [2].

The selectivity of PDT relies on two combined effects: the preferential retention of the sensitizer in the target tissue after systemic administration and the use of light focused on the target tissue [3].

Since sensitizers having a complete specific retention do not exist, the preservation of selectivity of this treatment requires the use as photosensitizers of compounds having a low cytotoxicity and a high phototoxicity [4]. Sensitizers having these characteristics, in fact, are able to act only on the cells of the target tissue which are the only ones to be illuminated. The normal body cells can incorporate the sensitizer but they can survive the treatment thanks to the low cytotoxicity of these "ideal sensitizers".

Among all classes of compounds proposed as sensitizers in PDT we have carried out a systematic study of the combined use of a natural chlorin, chlorophyll *a* (Chl *a*), with cyclodextrins (CD). Natural chlorins are pigments characterized by intense absorption bands in the 600–850 nm wavelength region, where there is the maximum depth of light penetration into tissues [5–7]. Cyclodextrins (CDs) are cyclic oligomers of glucose characterized by the ability to form inclusion complexes with a large number of organic molecules and are widely used in pharmacological applications as delivery systems of drugs having a poor solubility in water.

The main limitation of the use of Chl *a* was due to its very poor solubility in water and its high tendency to aggregate. CDs having different cavity size and moities have been considered as delivery systems of Chl *a* in solution and the obtained supramolecular systems have been characterized by different techniques [8–10]. The results of these studies have shown that some CDs are suitable carriers of Chl *a* in water and the Chl *a*/CD systems have properties which make them very promising as potential sensitizers in PDT.

Preliminary experiments aimed to test in vitro the efficiency of these systems in PDT have been recently performed and have evidenced that Chl *a*/CDs seems to act as sensitizer toward Jurkat cells [11].

In order to have a more detailed comprehension of this system, further investigations have been performed. The aggregation status of Chl *a* and the ability of four cyclodextrins, hydroxypropyl-β-cyclodextrin (HP-β-CD), hydroxypropyl-γ-cyclodextrin (HP-γ-CD), heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DIMEB), and heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin (TRIMEB), to solubilize the

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pigment in the cellular medium RPMI 1640 was estimated by means of UV–Vis absorption and static resonance light scattering (RLS) measurements. Finally, cell culture experiments with Jurkat cells have been performed in order to discriminate between cytotoxic and phototoxic effect of Chl *a*/CD.

2. Materials and methods

2.1. Chemicals

Hydroxypropyl-β-cyclodextrin (HP-β-CD) DS = 5.6, Hydroxypropyl-γ-cyclodextrin (HP-γ-CD) DS = 4.8, Heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DIMEB), Heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TRIMEB) and all solvents used were purchased from ALDRICH and used without further purification. Chlorophyll *a* was isolated from *Spirulina geitleri* and purified according to Omata et al. [12]. MTT kit test was purchased from Sigma.

2.2. Preparation of Chl a/CD solutions

All solutions were prepared mixing two stock solutions in ethanol (EtOH), one containing the CD and the other containing the Chl *a*. Then the mixture was dried under a stream of nitrogen and finally redissolved in a known amount of RPMI 1640 medium supplemented with 15% FBS. It was observed that for each CD, the amount of Chl *a* redissolved in the cellular medium at the end of this procedure was dependent on how long Chl *a* and CD remain in the mixed alcoholic solutions. Data reported in this paper refer to solutions prepared drying the EtOH soon after the mixing of the solutions.

The residual undissolved Chl *a* was recovered with ethanol, quantified by UV–Vis absorption measurements and used to correct the solution concentrations.

2.3. Physicochemical characterization of the Chl a/solution

UV–Vis absorption spectra were recorded using a Varian CARY/3 spectrophotometer. Static resonance light scattering (RLS) measurements were carried out using a Varian Cary Eclipse fluorescence spectrophotometer. RLS spectra were recorded with an excitation neutral density filter with 12.5% of transmittance.

2.4. Cell culture and incubation conditions

An immortalized cell line of human T-lynphocytes (Jurkat cells, ICLC, Italy) were cultivated in a RPMI 1640 medium supplemented with 15% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 I. U./ml penicillin (complete RPMI medium) [14–15]. Cells were cultivated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cell viability was determined by the trypan blue test. At this purpose aliquots of cells were incubated with 0.2% trypan blue and subsequently counted in a Thoma chamber.

In each well of a 96-wells culture plate 150 μl of cellular medium containing 100 000 cells were mixed with 150 μl of a CD or Chl *a*/CD solutions in the same medium having a concentration twice the final one: approximately 1 mM in CD and 1 10^{-5} M in Chl *a*. In the case of the Chl *a* without CD the pigment was solubilized in the medium using a 1% of ethanol. In order to test the cytotoxicity of CD, Chl *a* in ethanol 1% and Chl *a*/CD an MTT assay was performed at 24 and 48 h of cell culture [13]. Optical Density (O.D.) data from MTT tests were normalized to the O.D. value from MTT test obtained for the Jurkat cells in the complete RPMI medium having the same growth time.

In the phototoxicity experiments the wells containing the cells were illuminated after 24 h of incubation with the Chl *a*, CD or Chl *a*/CD and the cell viability was determined after further 24 h of growth always by MTT assay O.D. The illumination was performed positioning the 96-well cell culture on a plane having circular holes with a surface of 0.32 cm^2 each, corresponding to the surface of each single well. A LED with a 660 nm wavelength emission and a dissipation power of 50 mW was fixed below the plate in correspondance to the well. Solubilization, cytotoxic and phototoxic experiments were performed in triplicate.

3. Results and discussion

3.1. Solubilization of Chl a/CD in RPMI medium

The aggregation status of Chl *a* and the ability of CDs to solubilize the pigment in the complete RPMI medium was estimated by means of UV–Vis absorption spectra.

Chl *a* was solubilized in complete RPMI medium in presence of HP-β-CD, HP-γ-CD, DIMEB and TRIMEB 1 mM following the procedure described in the section Materials and Methods. In these experimental conditions the amount of Chl *a* solubilized in cellular medium is different for the four examined CDs. In particular it is evident from the absorbance value that, contrary to that observed in aqueous solution [8–10], the TRIMEB shows poor ability in solubilizing Chl *a* in the cellular medium. DIMEB and HP-γ-CD are able to carry in solution almost the same amount of Chl *a*. In particular the percentage of solubilized Chl *a* in the cellular medium results to be about 84.1% in HP-γ-CD and about 74.3% in DIMEB. The most effective is the HP-β-CD which solubilizes about 87.4% of Chl *a*.

Fig. 1 shows spectra of Chl *a* in solution of HP-β-CD, HP-γ-CD, DIMEB and TRIMEB 1mM dissolved in the medium used to grow the cells. The spectra are characterized by an intense Soret band at about 422 nm in the blue region of the visible spectrum and a Qy (0,0) band in the red region at about 663 nm. Similar to that observed in water [11] the peak in the red region shows an enlargement which indicates the presence of pigment aggregates [16,17]. In these spectra the aggregated species absorbing at 713 nm observed in aqueous solutions of HP-β-CD and HP-γ-CD and already evidenced in the water-rich region of binary water/organic solvent mixtures [18] is not evident. The presence of aggregated forms is also confirmed by measurements of resonance light scattering (RLS) shown in Fig. 2 which are a sensitive and selective probe to chromophore aggregation [19]. 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.

3.2. Cytotoxicity

Jurkat cells were grown for 24 and 48 h with the Chl *a*, CDs and Chl *a*/CDs dissolved in the complete RPMI medium to test the intrinsic toxicity of these systems.

The graphs of Fig. 3A and B show the MTT test results. Such results reflect the tolerance of cells towards Chl *a*, CDs and Chl *a*/CDs.

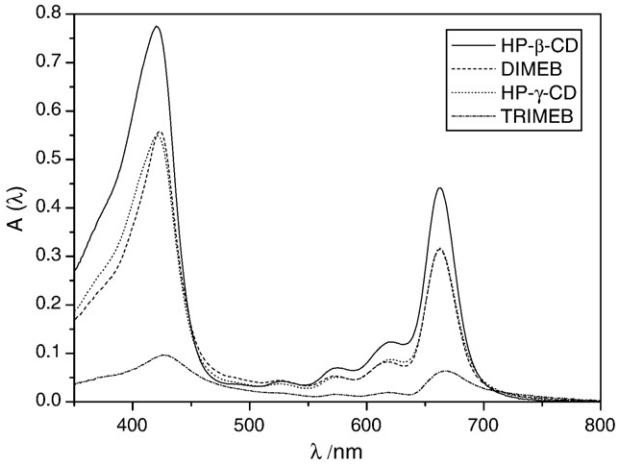

Fig. 1. Chl *a* absorbance, $A(\lambda)$, in the complete RPMI medium in presence of 1 mM CD *vs* λ.

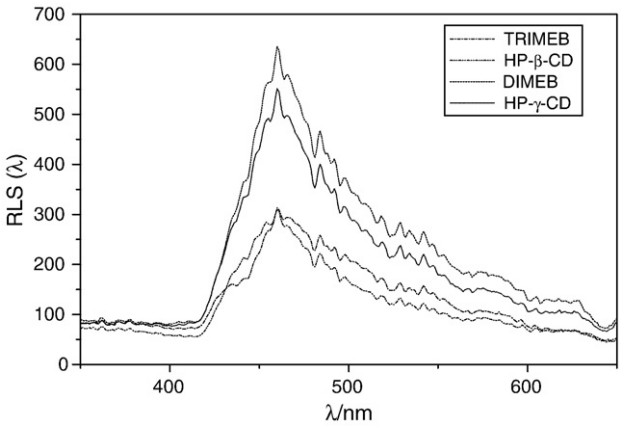

Fig. 2. Resonance light scattering, RLS(λ), profile of Chl *a* in the complete RPMI medium in presence of 1 mM CD *vs* λ.

After 24 h of incubation only in the case of TRIMEB an important citotoxicity towards Jurkat cells is observed. In fact the cell viability is reduced by about 30%. This effect disappears when the TRIMEB is in solution with the Chl *a*. DIMEB has an opposite behaviour to TRIMEB: the cyclodextrin alone does not reduce the cell survival, whereas the presence of Chl *a* induces a reduction in cell survival All other examined systems do not show any important reduction of the Jurkat cell viability.

After 48 h of incubation MTT results (Fig. 3B) show again how the behaviour of cells with TRIMEB and DIMEB is different from the other systems. DIMEB and Chl *a*/DIMEB exhibit the highest cytotoxicity. A comparable drop in the cell viability is also produced by TRIMEB whereas Chl *a*/TRIMEB is not cytotoxic at all.

The observed reduction of the cytotoxicity of TRIMEB induced by the presence of Chl *a* is probably due to the binding of these cyclodextrins with the pigment which makes them engaged and therefore less available to interact with the cellular membrane [20]. To understand the reasons for the behaviour of each cyclodextrin is not easy because they arise from the combination of more effects associated to the intrinsic ability of each CD to be cytotoxic and to interact with Chl *a* in the cellular medium.

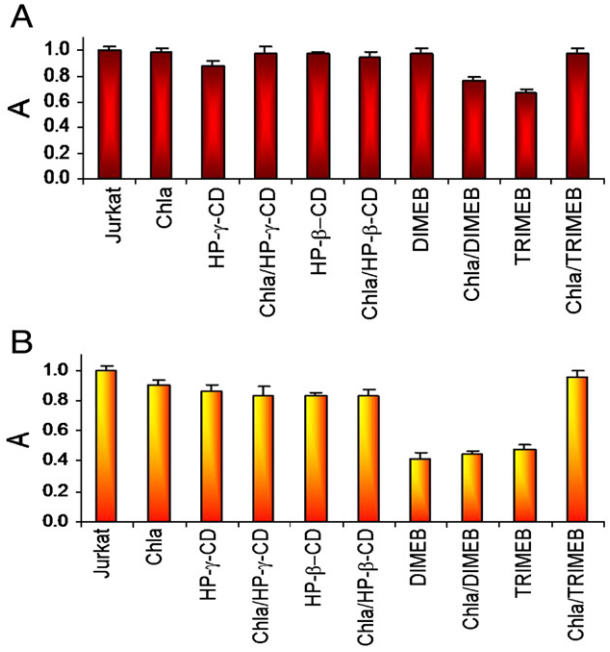

Fig. 3. Jurkat cell viability of 24 h (A) and 48 h (B) of growth in the complete RPMI medium, in presence of Chl *a* in a 1% of EtOH, CD and Chl *a*/CD evaluated by MTT assay.

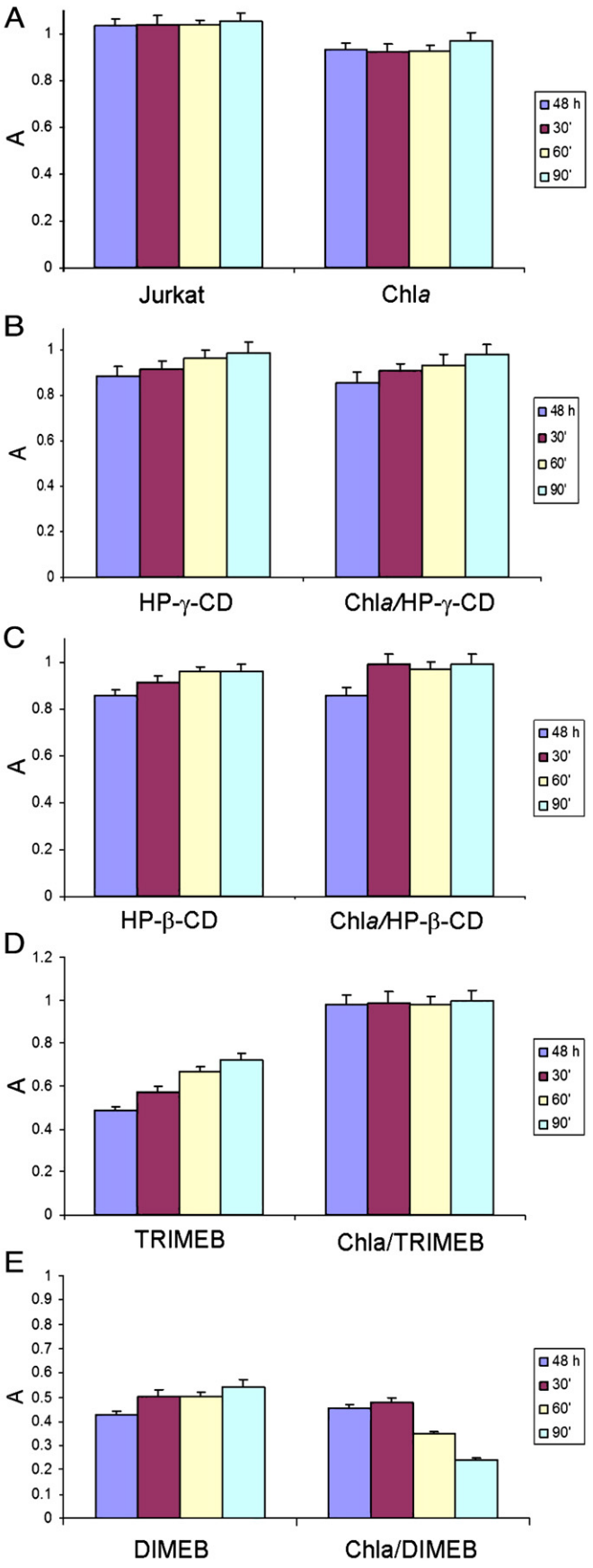

Fig. 4. Jurkat cell viabilities expressed as O.D. (MTT assay) in presence of Chl *a*, CDs and Chl *a*/CDs after different illumination time. The histograms 48 h indicate Jurkat cell viability after 48 h of incubation without illumination.

All these data indicate that cyclodextrin cytotoxicity does not only depend on their chemical nature but also depends on the contemporaneous presence in solution of a guest molecule. It is very important to be aware of this finding to evaluate correctly the change in the cell viability aimed to check the effectiveness of a sensitizer for PDT in presence of cyclodextrins.

3.3. Phototoxicity

In order to evidence the phototoxic effect and to discriminate it from the cytotoxic effect, the cell viabilities, expressed as O.D. (MTT assay), of Jurkat cells in presence of Chl *a*, CDs and Chl *a*/CDs were reported in the graphs (Fig. 4A–E). After illumination no decrease in the cell viability of Jurkat cells was observed as expected. A similar result was also obtained in presence of Chl *a* in EtOH 1%. It turns out that the illumination does not reduce or increases the cell viability. Furthermore the presence of Chl *a*, even though slightly cytotoxic, is not phototoxic (Fig. 4A right). The reduction in the cell survival reported in the previous study [11] might therefore be due to the intrinsic toxicity of the system.

The Jurkat incubated with HP-β-CD, HP-γ-CD, DIMEB and TRIMEB shows an increase in the cell viability after the illumination which raisesrises at increasing of the illumination time (Fig. 4B–E left).

An analogous result, but of lower entity to those observed in absence of the pigment, is also observed for HP-γ-CD in presence of Chl *a* (Fig. 4B right). In fact as already stated in the previous section the incubation of cells with HP-γ-CD, DIMEB and TRIMEB reduces their viability compared to the Jurkat cells grown in complete medium. After the illumination the reduction of the cell viability compared to the Jurkat cells is lower than that obtained without the illumination. It is possible to suppose that the illumination, probably producing a small increase in temperature, favours the binding of compounds present in the cell culture with CDs which become therefore less toxic for the cells.

Only cells incubated with Chl *a*/DIMEB show a significant decrease of viability at increasing of the illumination time which point out that this system is phototoxic. Taking into account that the study on the cytotoxicity has evidenced that the system Chl *a*/DIMEB is the most toxic, it is likely that the effectiveness of Chl *a*/DIMEB as sensitizer in PDT is also influenced by its intrinsic toxicity which weakens the cells.

4. Conclusion

The aggregation status of Chl *a* and the ability of CDs to solubilize the pigment in the cellular medium is different from that observed in water [8–11]. In particular TRIMEB, the permethylated cyclodextrin having the best ability to solubilize Chl *a* in water as monomer, is the one which works worst in the RPMI 1640 medium. In spite of the absence of phototoxicity obtained in the experimental conditions of illumination used in this study, the CD which has the best characteristics to form with Chl *a* a potential supramolecular system for PDT applications is HP-β-CD. This CD is in fact the most effective in the solubilization of Chl *a* in the cellular medium and also is that which has the lowest cytotoxicity. The results of biological experiments, in addition, indicate that cyclodextrin cytotoxicity does not only depend on their chemical nature but also on the contemporaneous presence in solution of compounds able to interact with them.

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