Available online at www.sciencedirect.com
ScienceDirect
Bioelectrochemistry 70 (2007) 39–43
Bioelectrochemistry
www.elsevier.com/locate/bioelechem

Cyclodextrin/chlorophyll *a* complexes as supramolecular photosensitizers

P.L. Dentuto [a,*], L. Catucci [a,b], P. Cosma [a,b], P. Fini [b], A. Agostiano [a,b], S. Hackbarth [c], F. Rancan [c], B. Roeder [c]

[a] *Dipartimento di Chimica Università di Bari Via Orabona 4, 70126 Bari, Italy*
[b] *IPCF-CNR, sez. Bari, Via Orabona 4, 70126 Bari, Italy*
[c] *Institute of Physics, Humboldt-Universität zu Berlin, Newton Str.15, 12489 Berlin, Germany*

Received 31 May 2005
Available online 5 April 2006

Abstract

The interactions between chlorophyll *a*, and three cyclodextrins, hydroxypropyl-β-cyclodextrin heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin and hydroxypropyl-γ-cyclodextrin, were studied in aqueous solutions by means of absorption, emission and circular dichroism spectroscopy. Nanosecond laser flash photolysis and steady-state singlet oxygen generation experiments were performed to clarify the photoactivity of chlorophyll *a* in these systems. Moreover the photosensitizing activity of these complexes towards human leukemia T-lymphocytes (Jurkat cells) was tested and compared with that of the free sensitizer, chlorophyll *a*. The results obtained indicate that each cyclodextrin is able to carry the pigment in monomeric form inside of cells producing singlet oxygen.

Keywords: Chlorophyll *a*; β- and γ-cyclodextrins; Photodynamic therapy; Jurkat cells

1. Introduction

The use of photosensitizers together with visible light is a widely studied and promising technique in the treatment of tumors (photodynamic therapy, PDT) [1]. The sensitizer should preferentially localize in the tumor tissues that will be irreversibly, directly or indirectly, destroyed by photogenerated singlet oxygen. In general light with wavelength in the red region of the visible spectrum is used to achieve a deeper penetration in the tumor tissue [2,3]. Then the sensitizer should exhibit intense absorption bands in this spectral region. Although a great variety of compounds can sensitize the formation of singlet oxygen in organic solvents and water, it is well known that sensitizers with absorption wavelengths longer than 800 nm often show a low photostability. Moreover the energy of the lowest excited triplet is too small for promoting phototoxic processes. Therefore, the PDT-literature deals mainly with dyes having intense absorption bands between 600 and 800 nm [4,5]. For this reason our research activities were focused on the photochemical and photophysical characterization of porphyrins and chlorines, which present absorption maxima in this wavelength region, with the aim to develop a new class of sensitizers for biomedical applications [6–9]. In particular, these studies concern the use of cyclodextrins for solubilizing hydrophobic dye molecules in aqueous environment. Cyclodextrins are cyclic oligomers of six (α), seven (β) or eight (γ) α-D-glucopyranose molecules linked by α-(1,4) bonds, with toroidal shape which present an inner hydrophobic cavity and an external hydrophilic surface. These characteristics allow the solubilization of hydrophobic molecules in aqueous solutions by host–guest inclusion complex formation [10]. The complexation generally affects the properties of the guests in the ground and/or excited states, i.e. their photophysical and photochemical parameters [11,12]. Our studies on the behavior of chlorophyll *a* (Chl *a*) in two β-cyclodextrins showed that the cyclodextrins form inclusion complexes solubilizing the dye molecules in water mainly in monomeric form, which is the photoactive species. In this note we report the photophysical properties of cyclodextrin/chlorophyll *a* complexes studied using both steady-state and time-resolved optical spectroscopy, with regard to their intracellular uptake and photosensitizing

* Corresponding author. Tel.: +39 080 5443443; fax: +39 080 5442128.
E-mail address: pdentuto@yahoo.it (P.L. Dentuto).

1567-5394/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bioelechem.2006.03.026

activity towards human leukemia T-lymphocytes (Jurkat cells). In particular the results of a study on inclusion complexes of chlorophyll *a* with cyclodextrins having different cavity size and different substituents are presented. The Chl *a* intracellular uptake has been verified and the results, compared with those obtained for free sensitizer, chlorophyll *a*, suggest that Chl *a*/CDs complexes may be a promising candidate for further use in PDT experiments.

2. Materials and methods

Hydroxypropyl-β-cyclodextrin (HP-β-CD) (DS=5.6), hydroxypropyl-γ-cyclodextrin (HP-γ-CD) (DS=4.8), heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD) and all other 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. [13]. Each solution was prepared mixing the appropriate amounts of alcoholic stock solutions of cyclodextrin and Chl *a*. The mixture was evaporated under a stream of dry nitrogen. The thin film of pigment and CD was redissolved in the desired quantity of bidistilled water. The residual undissolved Chl *a* was recovered with ethanol, quantified by UV–Vis absorption measurements and used to correct the solution concentrations. The tolerance of cells towards the cyclodextrin concentration was checked by XTT (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-5-[(phenylamino)carbonyl-2*H*-tetrazolium hydroxide]) kits. The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolic active cells [21]. Therefore, the described process can only occur in viable cells. The formazan dye formed is soluble in aqueous solutions and is directly quantified using a scanning multiwell spectrophotometer (ELISA reader). Concentration of 0.1 mM was used for TM-β-CD solutions, instead a concentration of 1 mM was used for HP-β-CD and HP-γ-CD solutions. Jurkat cells (Clone E6-1) were grown in suspension in RPMI 1640 medium supplemented with 10% FCS, bacteriostatica, and mycostatica in a humidified atmosphere of 5% CO_2 and 95% air. Cells were usually reseeded into fresh medium every second day. Cell proliferation and cell viability were determined by the trypan blue test. For this purpose, aliquots of cells were incubated with 0.2% trypan blue and subsequently counted in a Fuchs-Rosenthal chamber. Viability of cells was defined as percentage of living cells to the total cell number. Chl *a* or Chl *a*/CD were added to the growth medium 2 h after reseeding the cells into fresh medium. Chl *a* was administered in ethanol (0.05% final ethanol concentration), and Chl *a*/CD, in buffer Dulbecco phosphate (PBS). Before starting the incubation experiments, the tolerance of cells towards ethanol was checked. For the cell irradiation experiments, a special setup was used. After incubation with or without sensitizers, 1×105 cells in 100 μl per well were PBS washed twice and placed in a 96-well cell culture plate (Falcon). The 96-well culture was positioned on a plane having a circular hole with a surface of 0.32 cm^2, corresponding to the surface of each single well. A laser diode with a 668 nm wavelength emission was fixed below the plate in correspondence to the well. The out-put power of the laser trough the hole and a culture plate cover was of 0.60 mW, so that the irradiance resulted to be 2.12 mW/cm^2. Irradiation times of 180 s and 30 s were used, corresponding to doses of 400 and 64 mJ/cm^2. After the irradiation, fresh medium was added and cells were incubated at 37 °C, 5% CO_2 and 100% humidity prior further analysis. 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 the maximum absorption of Chl *a* in the Soret band. The circular dichroism spectra were recorded using a JASCO J810 spectropolarimeter. The measurements of steady-state singlet oxygen luminescence were performed in spectral range of 1200–1350 nm. A Versa Disk ELS laser was used to excite the samples at 515 nm and luminescence signal was recorded by a gallium arsenide pin diode and monochromator. Flash photolysis experiments were carried out with an ns-Nd-YAG laser (BMI) to excite the sample at 532 nm. Perpendicular to this excitation pulse, a cw-test beam was passed through the sample (XBO 100 with monochromator at 470 nm). The intensity of this test beam was monitored with a fast Si-PIN diode and recording oscilloscope (HP5415). Occupation of the lowest triplet state results in an additional absorption and hence a reduction of the transmittance of the sample for the test beam. The decay of this increased absorption is directly proportional to the relaxation of the lowest excited triplet state. For laser flash photolysis experiments the measurements were carried out after 24 h of incubation; the cells with Chl *a*/CD complexes were separated from the growth medium and washed twice with buffer.

3. Results and discussion

The tolerance of cells towards the cyclodextrin and Chl *a* concentration was checked by XTT test. This test allows the quantification of viable cells and therefore it has been used to determine the less toxic cyclodextrin and Chl *a* concentration. The obtained results indicate that the less non-toxic concentration are: 0.1 mM for TM-β-CD solutions, 1 mM for HP-β-CD and HP-γ-CD solutions (Fig. 1) and 10^{-5} M for Chl *a*. The absorption spectra of Chl *a*, in aqueous solution of HP-β-CD, TM-β-CD and HP-γ-CD, are shown in Fig. 2. The spectra are characterized by an intense Soret band in the blue region of the visible spectrum and a Qy (0,0) band in the red region at about 670 nm. The attention has been focused on the Chl *a* Qy transition because it is indicative of the presence of aggregated species in solution [14]. Enlargement and shift of this band, relevant to that of Chl *a* in ethyl ether, indicate the presence of dye dimers or larger aggregates [15–17]. In details the absorption spectra of Chl *a* in all cyclodextrins show the presence of a different aggregate distribution and evidence the presence of a particular aggregated species absorbing at 713 nm. This species has already been observed and characterized in the water-rich region of binary water/organic solvent mixtures [17]. In particular a “micelle-like” aggregate structure, with the chlorophyll phytyl chains anchored by hydrophobic interactions in the inside, and the macrocycles addressed toward the bulk water, has been supposed for this aggregate form [15]. The

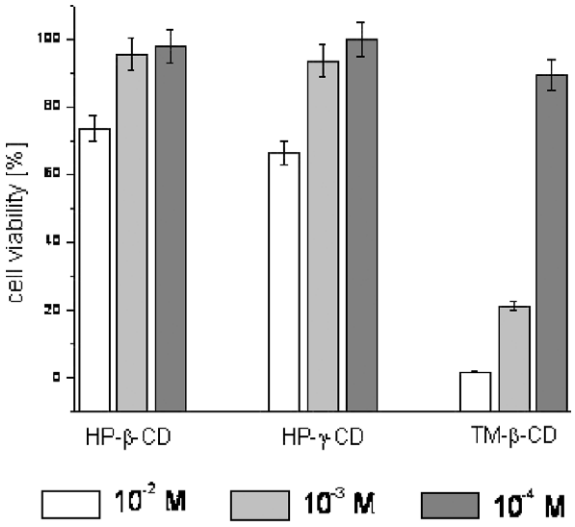

Fig. 1. Jurkat cell toxicity 24 h after incubation with HP-β-CD, HP-γ-CD and TM-β-CD.

presence of pigment aggregates in the investigated range of concentration was confirmed by absence of fluorescence signals (data not shown) and by circular dichroism measurements (Fig. 3). It is well known that chlorophyll aggregates do not show any detectable fluorescence emission because of an internal energy transfer between the porphyrin rings; moreover Chl *a* aggregates show a conservative peak in the red region of circular dichroism spectra [18]. In Fig. 3 the circular dichroism of Chl *a* in HP-β-CD, TM-β-CD and HP-γ-CD solutions is reported. The presence of a conservative red band characterized by a positive band at about 730 nm and a negative one at about 698 nm is evident for each system. This splitting confirms the presence of a strong coupling between the dipole transition of different Chl *a* molecules characteristic of aggregated form of the dye in aqueous system as reported by Agostiano et al. [19]. In particular the split signals show a different symmetry in the studied system although the zero crossing wavelength is 713 nm for all cyclodextrin solutions indicating that the prevalent aggregated form of Chl *a* is the species with the absorption maximum at 713 nm, in agreement with the absorption data. The non-symmetrical splitting of the two bands and their enlargement, observed in the case of HP-β-CD and TM-β-CD, indicate the presence of a non-uniform distribution of different Chl *a* aggregate in these systems. The increased symmetry of the split CD signal recorded for HP-γ-CD is in agreement with the higher amount of 713 absorbing species in this solution as observed in the absorption spectra. It is also possible to observe a corresponding increase of CD non-conservative peak at about 670 nm relative to the monomeric form of Chl *a* which is the prevalent form of the pigment in TM-β-CD solution.

The presence of these aggregate species gives rise to a dramatic reduction of photoinduced singlet oxygen generation and a decreased Inter System Crossing (ISC) quantum yield, as confirmed by the absence of steady-state singlet oxygen luminescence and the triplet Chl *a* decay signals with and without oxygen in all systems.

A completely different behavior was observed when Chl *a* and Chl *a*/CDs systems were added to the cells. Triplet decay traces, obtained by flash photolysis experiments, carried out with Chl *a* and Chl *a*/CD incubated cells, are reported in Fig. 4. Triplet decay times in air-saturated and nitrogen-flushed samples reported in Table 1 for Chl *a* and Chl *a*/CDs are nearly equal. This suggests that the interaction of the sensitizer first excited triplet state with oxygen is nearly the same for both Chl *a*/CDs and Chl *a* in the biological environment. Since it is well known [20] that a good sensitizer should have a long triplet decay time in the absence of oxygen and a short one in the presence of oxygen, these systems can be considered as promising sensitizers for applications in PDT.

To this aim the phototoxicity of Chl *a*/CD complexes (in water) was tested in comparison with the Chl *a* free (0.05% ethanol). Fig. 5 reports the phototoxicity of the studied compounds after different time of irradiation. The experimental

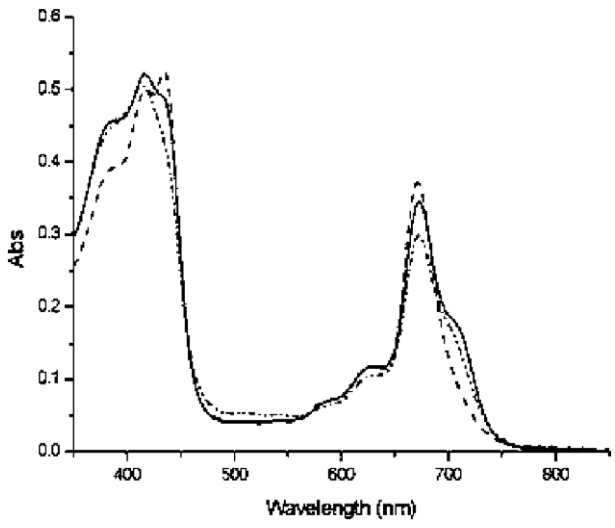

Fig. 2. Absorption spectra of Chl *a* 1×10^{-5} M in aqueous solution of HP-β-CD 1×10^{-3} M (– · · –), HP-γ-CD 1×10^{-3} M (—) and TM-β-CD 1×10^{-4} M (- - - -).

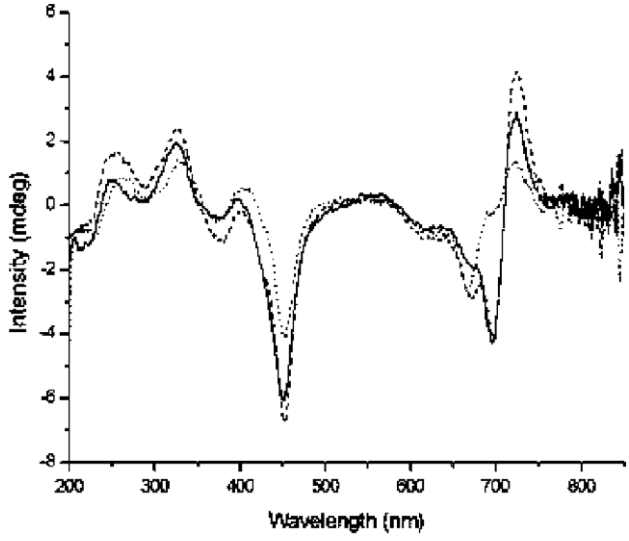

Fig. 3. Circular dichroism spectra of Chl *a* 1×10^{-5} M in aqueous of HP-β-CD 1×10^{-3} M (—), HP-γ-CD 1×10^{-3} M (- - - -) and TM-β-CD 1×10^{-4} M (·····).

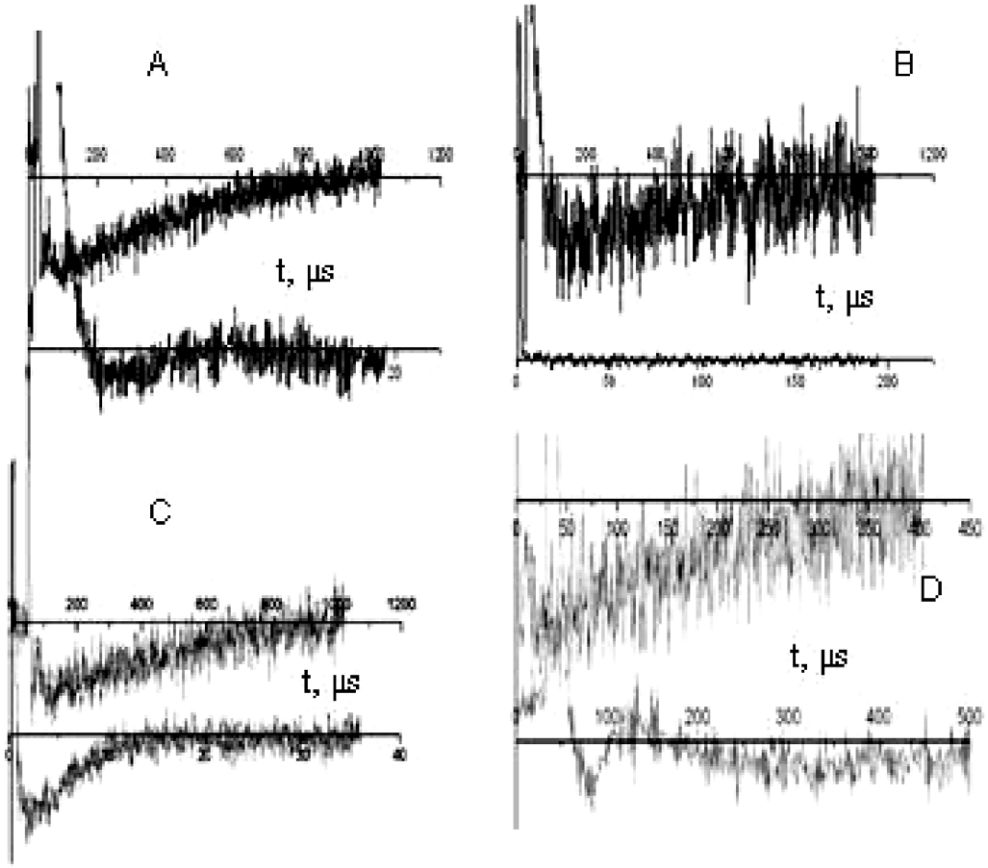

Fig. 4. Triplet decay of (A) Chl *a*, (B) Chl *a*/HP-β-CD, (C) Chl *a*/HP-γ-CD and (D) Chl *a*/TM-β-CD in (top) N_2-flushed and (bottom) air-saturated buffer.

data were obtained from air-equilibrated samples, 24 h after irradiation with a laser diode (688 nm, 2.12 mW/cm^2). As can be seen, after 180 s of irradiation an about 50% of cell mortality was recorded for cells incubated with Chl *a*/CD complexes. These results show that, even in a cellular environment, these complexes can induce the production of singlet oxygen.

4. Conclusions

In conclusion, the overall spectroscopic data presented in this study indicate that in aqueous solutions the fluorescence quantum yield of Chl *a*/CDs systems and the triplet and singlet oxygen quantum yield are dramatically reduced due to the presence of aggregate species. The behavior of these systems changes in presence of cells, suggesting that the interactions between Chl *a*/CD and cells favour the presence of the dye–CD complexes in its monomeric form, the only one able to photogenerate singlet oxygen. This behavior, together with the advantageous use of aqueous solution, makes the Chl *a*/CDs systems a promising candidate for further use in PDT experiments.

Table 1
Values of triplet decay time in the oxygen-depleted and in the air-saturated samples

Sample	Triplet decay time in the oxygen-depleted sample (μs)	Triplet decay time in the air-saturated sample (μs)
Chl *a* in cells	360	< 1
Chl *a*/HP-β-CD in the cell	334	4.5
Chl *a*/TM-β-CD in the cells	219	3.5
Chl *a*/HP-γ-CD in cells	283	< 1

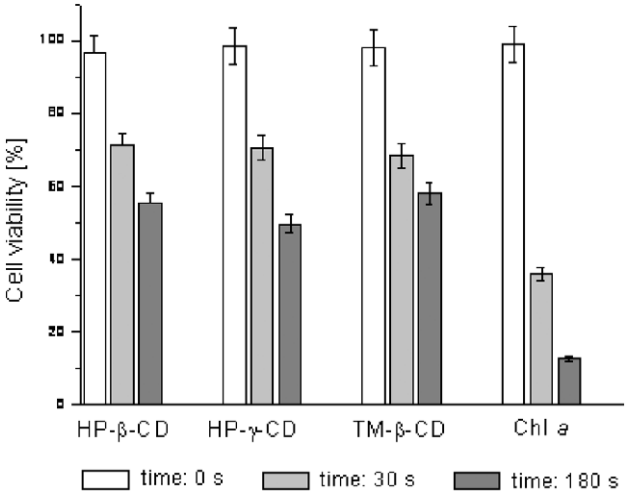

Fig. 5. Jurkat cell phototoxicity 24 h after irradiation with (grey) 400 mJ/cm^2 and (light grey) 64 mJ/cm^2 of red light (668 nm, 2.12 mW/cm^2).

References

[1] B.W. Henderson, T.J. Dougherty, Photodynamic Therapy: Basic Principles and Clinical Applications, Marcel Dekker, New York, 1992.

[2] R. Bonnett, Photosensitizers of the porphyrin and phthalocyanine series for photodynamic therapy, Chem. Soc. Rev. 24 (1995) 19–33.

[3] P. Lenz, In vivo excitation of photosensitizers by infrared light, Photochem. Photobiol. 62 (1995) 333–338.

[4] M. Aprahamian, S. Evrad, P. Keller, M. Tsuji, G. Balboni, C. Damge, J. Marescaux, Anti-Cancer Drug Des. 8 (1993) 101–114.

[5] R. Biolo, G. Jori, M. Soncin, B. Rihter, M. Kenney, M. Rodgers, Effect of sensitizer delivery system and irradiation parameters on the efficiency of photodynamic therapy of B16 pigmented melanoma in mice, Photochem. Photobiol. 63 (1996) 224–228.

[6] A. Agostiano, L. Catucci, P. Cosma, P. Fini, Aggregation processes and photophysical properties of chlorophyll a in aqueous solutions modulated by the presence of cyclodextrins, Phys. Chem. Chem. Phys. 5 (2003) 2122–2128.

[7] P.L. Dentuto, L. Catucci, P. Cosma, P. Fini, A. Agostiano, Photophysical and electrochemical properties of chlorophyll a-cyclodextrins complexes, Bioelectrochemistry 63 (2004) 117–120.

[8] P.L. Dentuto, M. Castagnolo, L. Catucci, P. Cosma, P. Fini, A. Agostiano, Chlorophyllide a/Cyclodextrin interaction in aqueous solution, J. Therm. Anal. Calorim. 77 (2004) 437–444.

[9] P.L. Dentuto, L. Catucci, P. Cosma, P. Fini, L. D'Accolti, A. Agostiano, C. Trevithick-Sutton, C.S. Foote, Effect of cyclodextrins on the physico-chemical properties of chlorophyll a in aqueous solution, J. Phys. Chem., B 109 (2005) 1313–1317.

[10] M.L. Bender, M. Komiyana, Cyclodextrin Chemistry, Springer-Verlag, West Berlin, 1978.

[11] V. Ramamurthy, R.G. Weiss, G.S. Hammond, A model for the influence of organized media on photochemical reactions, in: D. Volmar, G.S. Hammond, D.C. Neckers (Eds.), Advances in Photochemistry, John Wiley and Sons, New York, 1993, pp. 67–234.

[12] V. Balzani, F. Scandola, Supramolecular Photochemistry, Ellis Horwood, Chichester, England, 1991, pp. 288–299.

[13] T. Omata, N. Murata, Preparation of chlorophyll a, chlorophyll b and bacteriochlorophyll a by column chromatography with DEAE–Sepharose CL-6B and sepharose CL-6B, Plant Cell Physiol. 24 (1983) 1093.

[14] L. Fiedor, M. Stasiek, B. Mysliwa-Kurdziel, K. Strzalka, Phytol as one of the determinants of chlorophyll interactions in solution, Photosynth. Res. 78 (2003) 47–57.

[15] A. Agostiano, L. Catucci, G. Colafemmina, M. Della Monica, Chlorophyll a self-organization in microheterogeneous surfactant systems, Biophys. Chemist. 60 (1996) 17–27.

[16] A. Agostiano, P. Cosma, M. Della Monica, Formation of chlorophyll a photoreactive dimmers in alcoholic mixtures: spectroscopic and electrochemical study, J. Photochem. Photobiol., A Chem. 58 (1991) 201–213.

[17] A. Agostiano, P. Cosma, M. Trotta, L. Monsù-Scolaro, N. Micali, Chlorophyll a behavior in aqueous solvents. Formation of nanoscale self-assembled complexes, J. Phys. Chem. 106 (2002) 12820–12829.

[18] A. Agostiano, K.A. Butcher, M.S. Showell, A.J. Gotch, F.K. Fong, Dipole–dipole transfer between acetone solvates of chlorophyll a and chlorophyll a dehydrate dimmers in water/acetone mixture, Chem. Phys. Lett. 137 (1987) 37.

[19] A. Agostiano, L. Catucci, G. Colafemmina, H. Scheer, Role of functional groups and surfactant charge in regulating chlorophyll aggregation in micellar solutions, J. Phys. Chem. 106 (2002) 1446–1454.

[20] N.J. Turro, Molecular photochemistry, C and EN 8 (1967) 84–95.

[21] D. Gerlier, N. Thomasset, J. Immunol. Methods 94 (1986) 57–63.