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A spectroscopic study of photobleaching of Chlorin p6 in different environments

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Abstract

The photobleaching process of Chlorin p6 a photosensitizer used for photodynamic therapy was studied in neat phosphate buffer, phosphatidyl choline liposomes and in 10% fetal bovine serum. The full width half maxima (FWHM) of the soret band of Chlorin p6 increases in liposomes when compared to that in buffer and in 10% serum. This suggests that it may exist in aggregated form when bound to the lipid bilayer of the liposomes. The observed changes in the absorption spectra upon photoillumination are indicative of photomodification and/or photoproduct formation. A new nonfluorescent band was observed at 730 nm after photoirradiation. This band appears to arise due to aggregated species formed on photoirradiation. The rate of photobleaching of Chlorin p6 fluorescence in neat buffer and liposomes were comparable, but it was three times faster in the serum media. Those observations have been attributed to, negligible photobleaching rate of the aggregated species in liposomes and reduced diffusional motion of the drug when bound to serum proteins. Addition of histidine in serum medium reduces the photobleaching rate considerably while addition of mannitol has no effect on the rate. This indicates that the photobleaching of Chlorin p6 in these environments may proceed via a type II mechanism.

Keywords: Chlorin p6; Photodynamic therapy; Photobleaching; Photoillumination; Liposomes; Aggregation

1. Introduction

Photodynamic therapy (PDT), which is based on the interaction of light with certain chemical agents (photosensitizers) to generate cytotoxic species, is a rapidly expanding modality for treatment of cancer [1,2]. It is well known that most of the photosensitizers used for PDT are phototransformed on light illumination [3]. Such transformations are usually manifested as a decrease or "photobleaching" of fluorescence and/or absorbance of the photosensitizers. An understanding of the photobleaching of the photosensitizer and the resulting photoproducts is essential since these have significant influence on the photodynamic efficacy of the photosensitizer [1–4]. For example if the photosensitizer bleaches too rapidly, tumor destruction will not be complete. Further, the concentration and the bleaching rates determine photodynamic damage to normal tissue surrounding the tumor, which retains less sensitizer.

Chlorophyll derivatives Chlorin p6 and Chlorin e6 are gaining considerable attention as photosensitizers because of their strong absorption in the so-called therapeutic window (650–900 nm) and a high singlet oxygen yield [5]. In a recent study it was found that the rate of photobleaching of Chlorin e6 is almost three times in liposomes suspended in phosphate buffer compared to a neat solvent (dimethyl formamide). This difference was ascribed to the change in the polarity of the microenvironment around the sensitizer

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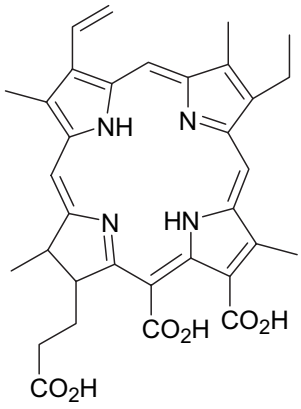
molecules [6]. We present in this paper results of a study on photobleaching of Chlorin p6 in neat phosphate buffer, phosphate buffer containing liposomes and phosphate buffer containing 10% fetal bovine serum. In those three environments although the bulk solvent is same i.e. phosphate buffer, the micro environments are heterogeneous. The results obtained show that while the rates of photobleaching in buffer and in liposomes are similar, the rate increases almost three times in the serum media. The results suggest that this arises because of the aggregation and binding of the drug to the hydrophobic environments in liposomes and serum media.

2. Materials and methods

Chlorin p6 was synthesized as described in literature [7] and purified by thin layer chromatography. L-α-Phosphatidyl choline from Sigma Chemicals (USA), fetal bovine serum from High Media (India) and bovine serum albumin (97%) from BDH Biochemicals (England) was used as received. Unilamellar liposomes having an average diameter of 1 μm were prepared in phosphate saline buffer following the procedure previously described [8]. A stock solution of Chlorin p6 is prepared in methanol (0.8 mM). Chlorin p6 samples in buffer, liposomes and in 10% serum were prepared by adding 25 μL of the stock solution to 3 mL of each. Photobleaching process of Chlorin p6 was measured in three environments, phosphate saline buffer (PBS), phosphatidyl choline liposomes (suspended in PBS) and in 10% (v/v) fetal bovine serum in PBS. For studies on photobleaching a 3 mL solution was taken in a quartz cuvette equipped with a magnetic stirrer. The OD of the three solutions was matched at the respective soret peak before photoillumination. Photoirradiation was carried out at 400 nm using a commercial Xe lamp source. The source bandwidth (FWHM) and power were 40 nm and 6 mW, respectively. The spot size of the blue light on the sample was 1 mm × 10 mm leading to an irradiance of 60 mW cm^{-2}. Absorbance spectra were recorded at 10 min intervals over a one-hour period. The kinetics was followed by monitoring the decrease in the soret band maxima of Chlorin p6.

3. Results and discussions

Fig. 1 shows the chemical structure of the photosensitizer Chlorin p6. In Fig. 2 we show the measured absorption spectra of Chlorin p6 in neat buffer, liposomes and in 10% FBS. For the same stoichiometric amount of the drug the absorbance of the soret band was slightly lower and showed a red shift in liposomes and serum compared to that in neat buffer. The position of the Q-band also undergoes red shift when the drug is



chlorin p6

Fig. 1. Chemical structure of Chlorin p6.

in liposomes or serum. The effect of solvent polarity on the absorption spectrum is described in the inset of Fig. 2. It is clear that the solubility of the drug is substantially lower in the nonpolar solvent hexane compared to the polar methanol. Porphyrin derivatives, like *meta*-tetra (hydroxyphenyl) chlorin which are hydrophobic in nature, show strong reduction in absorption intensity along with a broadening of all absorption bands in buffer [9,10]. This has been attributed to the aggregation of the drug in the polar environment. The presence of proteins (as for example in serum media) leads to monomerization. The presence of three ionizable carboxylic groups in Chlorin p6 (Fig. 1) suggests that it will be amphiphilic in nature. The reduction of absorption in hexane might be due to low solubility and/or due to aggregation. The absorption parameters for the drug in different environments are summarized in

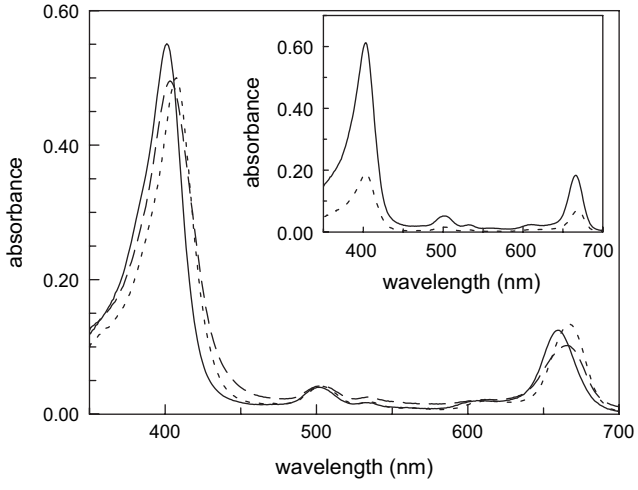


Fig. 2. Absorption spectra of Chlorin p6 (6.5 μM) in buffer (solid line), 10% FBS (dotted line) and in liposomes (dashed line). Inset: Absorption spectra in methanol (solid line) and in *n*-hexane (dotted line).

Table 1. From Table 1 it can be seen that there is a considerable increase in FWHM of the soret band in liposomes, but in hexane the increment is moderate/nominal. Thus in hexane the reduction of absorption may be solely due to the non-solubility of the drug due to its amphiphilic nature whereas in liposomes the drug may also exist in a state of aggregation in the lipid bilayer as suggested by the increase in FWHM of the soret band.

Fig. 3 describes the effect of photoillumination on absorption spectrum in the three different media, neat buffer, liposomes and in serum. The inset shows the absorption difference spectra (absorption after illumination minus absorption before illumination). Photoirradiation is seen to result in decrease in absorbance at several bands; around 400 nm (soret band), 660 nm (Q-band) and at ~500 nm. Also one can note an increase in absorption centered around 450 nm, 580 nm (seen only in PBS and in 10% FBS), 730 nm and in the near UV region. The ordinate scale has been expanded to highlight the photoirradiation induced increase in absorption observed in these bands. These changes in absorbance are indicative of photomodification and phototransformation upon photoillumination. The photoproducts have not yet been identified. However, in porphyrin, hematoporphyrin and chlorine derivatives, similar photoillumination induced decrease of absorbance of the soret and Q-band and an increase in absorbance in UV region and at 450–500 nm has been reported [11–14]. This would suggest that at least some of the photoproducts may be identical. An important finding of this study is the appearance of a new band at 730 nm in all the three environments. The species responsible for the 730 nm band was verified to be nonfluorescent. In an earlier study carried out by our group [7] it was found that aggregation of Chlorin p6 becomes effective below pH 5 and the aggregated species with absorption band at 675 nm was nonfluorescent. Concomitant with the formation of the aggregated species a blue shift of the absorption spectrum was also observed. In our experiments the pH of the medium was 7.4. Therefore, appearance of a red shifted nonfluorescent absorption band at 730 nm on photoirradiation may suggest photoirradiation induced aggregation of Chlorin p6.

Table 1
Absorption parameters of Chlorin p6 in different environments[a]

Solvent	Soret maxima (nm)	Q-band maxima (nm)	FWHM of soret band (cm^{-1})
Methanol	403	666	2208
n-Hexane	403	669	2335
Buffer	401	659	2172
Liposome	403	666	2522
10% FBS	408	667	2097

[a] In all cases the concentration of Chlorin p6 was 6.5 μM.

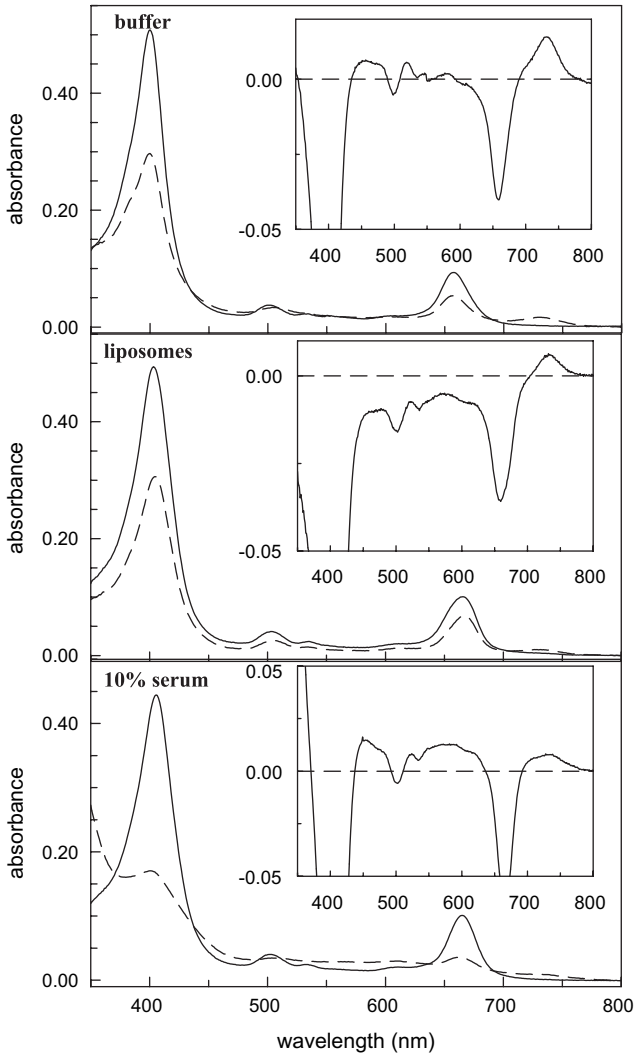


Fig. 3. Effect of photoillumination on the absorption spectrum of Chlorin p6 in different environments. The solid and dashed lines represent absorption before and after illumination. The inset in all the three panels shows the absorption difference spectrum. See Section 2 and Section 3 for more details.

Fig. 4 shows the rate of change of the absorbance of the soret band with irradiation time for the three medium. For all the cases the photobleaching follows first order kinetics. However, as seen in Fig. 4, for 10% FBS the photobleaching rate was faster ($3.7 \times 10^{-4}\,s^{-1}$) than that in neat PBS ($1.4 \times 10^{-4}\,s^{-1}$) or in liposomes ($1.2 \times 10^{-4}\,s^{-1}$). It has been shown that the rates of photobleaching of hematoporphyrin, photofrin are sensitive to solvent changes [15], thus solvent effects is one possibility. The origins of the solvent effects on the rates are however, not yet clearly understood but it is apparent that the rate depends upon the polar nature of the solvent. It is possible that more polar solvents are stabilizing the polar/dipolar transition states involved in the reaction with the singlet oxygen or other reactive oxygen species generated. In our case we see that the rate is comparable in neat PBS and in liposomes

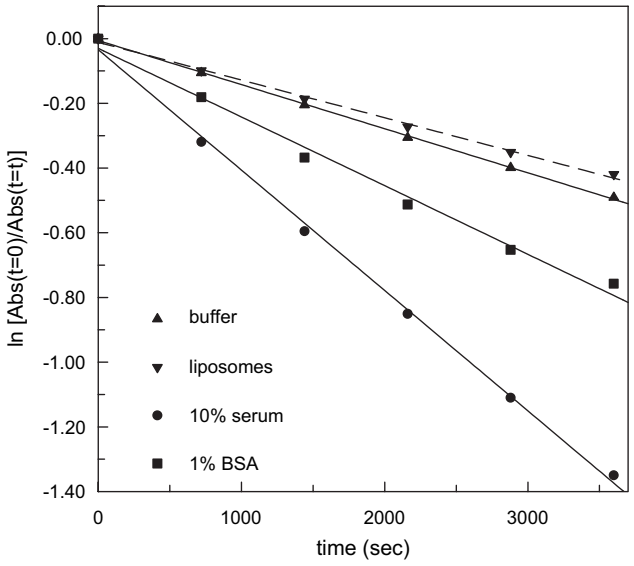


Fig. 4. Rate of change of soret band absorbance of Chlorin p6 upon photoillumination in different environments.

solubilized in PBS but it is three times faster in serum. In both liposomes and serum there are hydrophobic regions (bilayer of the lipid membranes and hydrophobic pockets in the serum proteins) distributed homogeneously inside the bulk solvent. The red shift of the absorption bands of the drug when going from neat buffer environment to liposomes and serum suggests that there is a partitioning of the drug between bulk water and the lipid bilayer or the protein matrix. In the lipid bilayer of the liposomes increase of the FWHM of the soret band (Table 1) suggests that Chlorin p6 maybe in a state of aggregation. However, this observed difference is not significant in the sense that we do not see any noticeable change in the rate of photobleaching in liposomes. This may be due to the fact that the aggregated species do not photobleach as efficiently as the monomeric species or the number density of the aggregated species in the lipid bilayer is much smaller than that of the monomeric species in the bulk. In the case of the serum solution the drug which is partially bound to the serum proteins and hence in a nonpolar or hydrophobic environment should photobleach at a lesser rate than in neat buffer. The quantum yield and singlet lifetime of Chlorin p6 in ethanol and buffer are 0.1, 3.2 ns and 0.17, 3.3 ns, respectively [16]. From these values the nonradiative rates for Chlorin p6 in ethanol and in buffer can be obtained using the relations:

$$\phi_f = k_r \tau_f \text{ and } k_r + k_{nr} = 1/\tau_f$$

where ϕ_f is the quantum yield, k_r and k_{nr} are the radiative and nonradiative rates and τ_f the radiative lifetime. The values of k_{nr} thus obtained ($28 \times 10^7\ s^{-1}$ for ethanol and $25 \times 10^7\ s^{-1}$ for buffer) show that the nonradiative rates (which also includes intersystem crossing rate) does not change significantly in going from the polar buffer to nonpolar ethanol. Hence it is unlikely that in serum solution the intersystem crossing rate will be increased significantly compared to that in neat buffer. In order to offer a possible explanation for the observed increase in photobleaching rate by a factor of 3, we propose that the drug binds to the surface of the proteins which in turn slows down its diffusional motion (which will now be equal to that of the protein). This might be the reason for its faster photobleaching because singlet oxygen/reactive species generated after photoillumination will now have a greater probability to react with the drug due to its lowered diffusional motion. It is pertinent to note that the drug bound to the liposomes would also have reduced diffusional motion which should increase its photobleaching rate. However, as discussed in the beginning of this section the observed broadening of the soret band of the drug provides evidence that in liposome solution, part of the drug is present in aggregated form. Therefore the observation that the photobleaching rate in liposomes is similar to that in buffer would suggest that photobleaching of the aggregates is not significant with respect to that of the mononmeric species. The rate of photobleaching of Chlorine p6 in PBS was found to increase by a factor of 2 on addition of 1% BSA (Fig. 4). This observation further confirms the possibility that binding to proteins and the concomitant reduction in diffusion rate may be responsible for the faster photobleaching observed in the serum media.

In order to determine the mechanisms responsible for photobleaching, mechanistic studies were carried out using known quenchers for singlet oxygen and free radicals. Fig. 5 shows the rates in 10% FBS with the

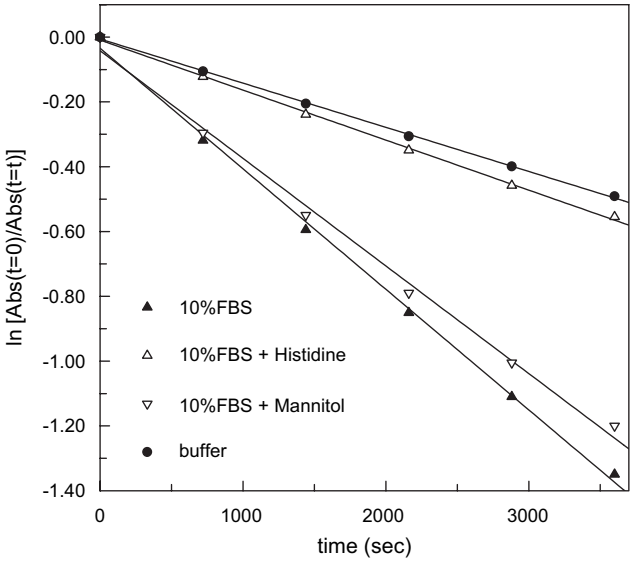


Fig. 5. Rate of change of soret band absorbance of Chlorin p6 upon photoillumination in different environments and effect of addition of histidine and mannitol. See Section 3 for details.

addition of histidine a well known quencher of singlet oxygen and mannitol which is a quencher for the radical species generated by type I process. It is evident from Fig. 4 that addition of 10 mM mannitol has little or no effect whereas addition of 1 mM histidine slows down the photobleaching rate. In presence of histidine the photobleaching rate is almost comparable to that in neat buffer. This would suggest that photobleaching in Chlorin p6 is primarily governed by singlet oxygen species.

4. Conclusions

In conclusion we have found out that Chlorin p6 due to its amphiphilic character solubilizes readily in polar solvents but in nonpolar solvents its solubility is reduced. In liposomes absorption spectrum suggests that it might exist in a state of aggregation in the lipid bilayer region. The rate of photobleaching is faster in serum when compared to neat buffer and liposomes. This has been explained on the basis of formation of aggregated species in lipid bilayers which does not photobleach efficiently and reduced diffusional mobility of the drug when it is bound to the proteins. Mechanistic studies on the rate of photobleaching are suggestive of a type II process. Photoillumination may also produce the aggregated species as suggested by the absorption spectrum after photoillumination. Since aggregated Chlorin p6 is hydrophobic and thus can be taken up more readily by cells, our results suggest that photoillumination of Chlorin p6 might lead to enhanced cellular uptake of the drug. Further studies are necessary to identify the photoproducts and the role of aggregated Chlorin p6 on cellular uptake as well as in PDT.

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