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Photobleaching of chlorins in homogeneous and heterogeneous media

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Abstract

Comparative spectroscopic studies and quantum chemical calculations involving sensitizers chlorin (C), meso-tetraphenyl chlorin (MTPC), octaethyl chlorin (OEC), pheophorbide a (PPa) and chlorin e6 (Ce6) have been performed. Upon irradiation, all except chlorin bleached detectably in a homogeneous system (DMF). The order of photobleaching efficiencies was Ce6 > PPa > OEC > MTPC > C. Combining these results with data from quantum calculations suggest that the efficiency of photobleaching is determined by the substituent groups in the photosensitizer. In a heterogeneous system (DPPC liposomes), however, only Ce6 photobleached significantly. This might be caused by the change of polarity of the environment around sensitizer molecules. In addition, the effects of additives that enhance or decrease photobleaching rates have also been assessed. The results suggest that the mechanisms of sensitizer photodegradation are complex and media-dependent.

Keywords: Chlorins; Photobleaching; Chemical structure; Singlet oxygen; Superoxide anion

1. Introduction

In the early 1990s, the first photodynamic therapy (PDT) sensitizer, Photofrin® (porfimer sodium) was approved by health boards in Canada, Japan, The Netherlands and the United States for use against cancers involving esophageal and endobronchial tumors, gastric carcinoma and bladder carcinoma [1]. In spite of the favorable results obtained with Photofrin®, especially in the treatment of superficial and early-stage neoplasias, some important factors still limit the efficacy of PDT, including its heterogeneous and partially unknown chemical composition, its low extinction coefficient in the red spectral region, and its relatively poor selectivity towards tumors versus most peritumoral tissues and normal tissues [2]. This is undoubtedly encouraging the accelerated development of second-generation photosensitizers such as chlorins and reduced porphyrins. These sensitizers have a definite composition and a strong absorption in the red spectral region [3,4]. New tumor-photosensitizing chlorins are sought which overcome or minimize the present drawbacks of PDT utilising Photofrin®.

On irradiation, most of the photosensitizers used in biomedical studies are degraded. In this process, the photosensitizers are probably attacked by the singlet oxygen they produce, although free radicals may also play a role [5,6]. This process, usually called "photobleaching", causes a decrease in absorption and fluorescence

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intensity. In principle, the photobleaching of sensitizers used in the treatment of solid tumors can be either a disadvantage or a advantage. If the sensitizer is bleached too rapidly during irradiation, the tumor may not be destroyed completely [7]. On the other hand, photobleaching a sensitizer can reduce its concentration in the body, especially in the skin, and decrease the level of irreversible damage to normal tissues [8]. Photobleaching also effects PDT clinical dosimetry, as the initial sensitizer concentration in tissues decreases on irradiation [9].

More information about the photobleaching behavior of sensitizers is needed to calculate the optimum PDT dose. This means that a study of the photobleaching of sensitizers can provide a scientific basis for drug screening and PDT dose calculations.

Since sensitizers exhibit different photobleaching behavior in solutions, cells and tissues, we studied the photobleaching characteristics of five chlorins (Chlorin (C), Meso-tetraphenyl Chlorin (MTPC), Octaethyl Chlorin (OEC), Pheophorbide a (PPa) and Chlorin e6 (Ce6)) in homogeneous and heterogeneous media.

2. Experimental

2.1. Reagents

Chlorin (C), meso-tetraphenyl chlorin (MTPC), octaethyl chlorin (OEC), pheophorbide a (PPa) and chlorin e6 (Ce6) were purchased from Porphyrin Products (Logan, UT). Phosphatidylcholine dipalmitoyl (DPPC), sodium azide (NaN_3), superoxide dismutase (SOD) and catalase were from Sigma Chemical Co. (St. Louis, MO). Methylviologen (MV^{2+}) was from Koch-Light Laboratories Ltd. (Colnbrook, Berks., England). Other compounds were commercial materials of the highest available purity (Fig. 1).

2.2. Methods

2.2.1. Preparation of liposomes

DPPC liposomes were prepared following the injection method described previously [10]. DPPC was dissolved in ethanol and the ethanolic solution was added to phosphate-buffered saline (PBS) (6.4 mmol/l Na_2HPO_4, 1.4 mmol/l KH_2PO_4, 137 mmol/l NaCl and 2.6 mmol/l KCl, pH 7.4) at 50°C with stirring. The rate of addition was 0.1∼0.05 ml/min. This process afforded unilamellar vesicles of DPPC. Chlorins were added to liposomes at this stage. The concentrations of chlorins were 1.2×10^{-5} mol/l.

2.2.2. Photobleaching measurements

A 2.0 ml sample of a reaction mixture was placed in a 10×10 mm quartz cuvette and irradiated using a red light treatment instrument (produced by Institute of Electronics, Academia Sinica), whose output has the spectrum containing $>90\%$ red light of $600 \sim 700$ nm and $<10\%$ infrared light of 700∼4000 nm. The incident light fluence rate was determined to be 25 mW/cm^2 by BTY-8204 radiometer (Institute of Solar Energy in Beijing). The spectra ($300 \sim 800$ nm) of samples were recorded on a Hitachi U-3200 UV/visible spectrophotometer at $20 \sim 22$°C.

The efficiency of photobleaching of a sensitizer is strictly related to the incident illumination dose (D), which is the production of the incident light fluence rate (I) and illumination time (t). The optical density of the sensitizer at the illumination wavelength defines the absorbed part of the radiation. Different sensitizers of equal concentration have different optical densities at the illumination wavelength, and this was taken into account when their photobleaching efficiencies were compared. The light source used in this study was not monochromatic light, but mainly ($>90\%$) red light containing $600 \sim 700$ nm. The areas below the absorption curves of sensitizers in the range of $600 \sim 700$ nm were used to represent the size of absorption of sensitizers following irradiation. The bleaching of the absorption differed in magnitude depending on the wavelength at which it was measured. In order to eliminate this dependence, we used relative absorption bleaching values obtained by dividing ($\Delta(\lambda_{max})$ by the value of the initial absorption $A(\lambda_{max})$ at the peak wavelength in the red region. (The absorption peaks of sensitizers in the red region are important to PDT. Red light is widely used due to its ability

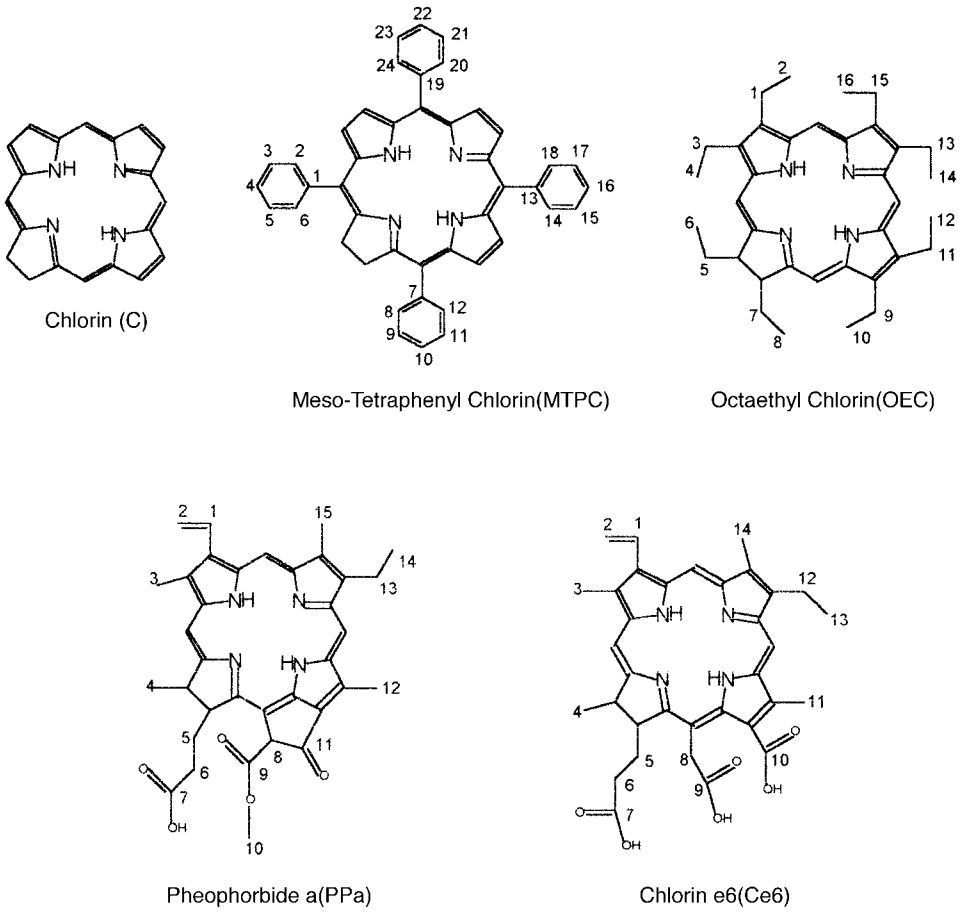

Fig. 1. Chemical structures of chlorins.

to penetrate tissues [11]). The initial aborption bleaching rate constants (k_{max}) for chlorins were evaluated by the following formula [12]

$$k_{max} = \frac{1}{D}\frac{1}{S}\frac{\Delta A(\lambda_{max})}{A(\lambda_{max})}$$

where D is the illumination dose, S is the integrated absorbance area of sensitizer in the range of 600 ~ 700 nm, $\Delta A(\lambda_{max})$ is the absorption difference at the peak wavelength (λ_{max}) in the red region after illumination and $A(\lambda_{max})$ is the initial absorption value at λ_{max}.

2.2.3. Theoretical methods

The SCF-MO-AM1 [13] calculations were performed on the five chlorins employing the MOPAC 7.0 program package. Molecular geometries obtained from molecular mechanics calculation using MMX [14], an enhanced version of MM2 [15], in the PCMODEL V 6.0 program package were employed as starting geometries for quantum calculations. Stable geometries were obtained using an energy gradient method [16].

3. Results and discussion

3.1. Experimental studies

3.1.1. Photobleaching in a homogeneous medium

The photobleaching of chlorins in a homogeneous medium (DMF) was studied, no new distinctive absorption peaks appeared in the spectral

region 300 ~ 800 nm of any chlorin after irradiation. Except for chlorin the absorption peaks of chlorins in the region underwent photobleaching on irradiation. The kinetics of photobleaching and initial bleaching rate constants are presented in Fig. 2 and Table 1. The order of photobleaching efficiencies was Ce6 > PPa > OEC > MTPC > C.

It is believed that photobleaching of a sensitizer is caused by $^1O_2$ attack, although free radicals may be involved [5,6,17,18]. In order to investigate what roles these active species play in the photodegradation of chlorins, we examined the effects of N_2, the electron acceptor methylviologen (MV^{2+}) and NaN_3, an effective quencher of singlet oxygen ($kq = 5.8\times10^8$ m^{-1} s^{-1}) [19], on the photobleaching of chlorins in DMF (Table 2).

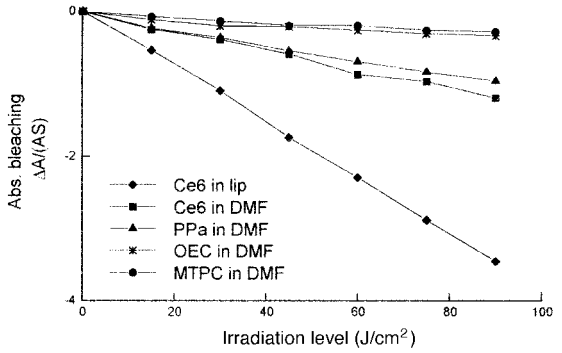

Fig. 2. Kinetics of absorption of the photosensitizers.

According to Table 2, NaN_3 had an obvious inhibition effect on the bleaching of chlorins in DMF, especially on that of OEC and MTPC, suggesting that singlet oxygen played an important role in the presence of oxygen.

Under nitrogen, the photobleaching rates of PPa, OEC and MTPC decreased to some extent, which is similar to the results with HPD [20]. This also suggested that photobleaching was associated with oxygen. To our surprise, however, Ce6 underwent photobleaching at almost at the same rate in the presence and absence of oxygen. This suggested that Type I processes (nonoxygen radicals) could not be ignored in the photodegradation of Ce6, especially in deaerated solutions. To find out the reasons causing the high bleaching efficiency of Ce6 under nitrogen, we examined the effects of the effective quencher of hydroxyl radical mannitol [21] on bleaching of this compound under nitrogen. The results showed that bleaching rate decreased by 20% in the presence of mannitol (5 mM), consequently, a possible path for photodegradation of Ce6 in anaerobic solution was developed:

$$Ce6 + Ce6^* \rightarrow Ce6^{\bullet+} + Ce6^{\bullet-} \quad (1)$$

$$Ce6^{\bullet+} + H_2O \rightarrow Ce6H^+ + {}^{\bullet}OH \quad (2)$$

$$Ce6 + {}^{\bullet}OH \rightarrow \text{products} \quad (3)$$

Table 1
Rate constants of initial absorption bleaching for chlorins in DMF

	Chlorin	MTPC	OEC	PPa	Ce6
λ_{max} (nm)	635	652	644	667	664
k_{max} ($\times10^{-3}$ J^{-1} cm^2)	~0	4.63	6.89	12.30	13.27

Table 2
Relative rate constants of bleaching chlorins in DMF under different conditions

Conditions	Ce6	PPa	OEC	MTPC
Normal condition	1	1	1	1
NaN_3 (14 mM)	0.54	0.28	0	0.04
30 min nitrogen bubbling before irradiation	1.05	0.63	0.07	0.27
MV^{2+} (0.03 mM)	15.77	0.98	0.85	0.38

This indicates that $^{\bullet}OH$ is involved in photobleaching of Ce6 under nitrogen.

As superoxide ion is known to be formed by irradiation of chlorins, it was possible that the photobleaching of chlorins could arise in part from an attack of this species on chlorin radical cations [18]. An indication of the role of superoxide in the photooxidation of chlorins was obtained by irradiating chlorins in the presence of the electron acceptor methylviologen (MV^{2+}). This compound should quench excited states of chlorins by the electron transfer indicated in Eq. (4) [21]. Subsequent reaction of reduced viologen to yield superoxide [Eq. (5)] should occur as has been demonstrated in previous studies [22,23]. Thus, the use of viologen would provide an approach to studying the reaction between radical cations of chlorins and superoxide [Eq. (6)] [20].

$$S^* + MV^{2+} \rightarrow S^{\bullet+} + MV^{\bullet+} \quad (4)$$

$$MV^{\bullet+} + O_2 \rightarrow MV^{2+} + O^{\bullet-}_2 \quad (5)$$

$$S^{\bullet+} + O^{\bullet-}_2 \rightarrow \text{products or } P + O_2 \quad (6)$$

The results showed that while the photobleaching of PPa, OEC and MTPC was retarded by the addition of viologen, bleaching of Ce6 was enhanced significantly (Table 2). Since the generation of a large amount of $^1O_2$ could be ruled out under these conditions [18], it is possible to conclude that $O^{\bullet-}_2$ might not be ignored in the photobleaching of chlorins and in fact might increase the photooxidation of Ce6 greatly.

3.1.2. Photobleaching in a heterogeneous medium

To mimic the photobleaching behavior of chlorins in biological systems, we investigated their bleaching in DPPC liposomal media. The results are presented in Table 3 and Fig. 2. While Ce6 photobleached appreciably, PPa, OEC, MTPC and C showed no detectable bleaching. The effects of N_2, NaN_3, D_2O, SOD, catalase and mannitol on the bleaching of Ce6 in DPPC liposomes was investigated, and the results are presented in Table 4. Based on the results, at least two major reaction pathways are indicated. The first involved singlet oxygen generation and attack on ground-state Ce6, which was supported by an increase in bleaching rate in DPPC liposomes prepared with 70% D_2O and the inhibition effects of NaN_3 on Ce6 degradation. The second pathway appears to involve electron transfer from excited Ce6 to generate superoxide and the Ce6 radical cation and interaction of the these two species. In addition, $^{\bullet}OH$ generated from $O^{\bullet-}_2$ via Fenton reaction might mediate Ce6 degradation.

The effects of SOD and catalase on Ce6 bleaching rate were surprising. It is known that catalase catalyzes the dismutation of hydrogen peroxide to water and oxygen, while SOD catalyzes a reaction in which superoxide is converted to oxygen and hydrogen peroxide. It can be seen from Table 4 that SOD and catalase had positive effects on Ce6 bleaching, which corresponds to the results of the effects of SOD and catalase on the photodegradation of porphyrins in a prior report [5]. However, the mechanism(s) for increased photobleaching rates caused by SOD and catalase are still not known. In addition, mannitol, which quenches free radicals such as hydroxyl radical [12], showed an inhibition effect on Ce6 photobleaching, suggesting that hydroxyl radicals were involved in the photobleaching process of Ce6 in DPPC liposomes.

Comparing Table 4 with Table 3, it is clear that NaN_3 decreased the bleaching rate of Ce6 much more in DMF (46%) than in DPPC liposomes (8%). In a liposomal system, NaN_3 is dissolved in

Table 3
Rate constants of initial absorption bleaching for chlorins in DPPC liposomes

	Chlorin	MTPC	OEC	PPa	Ce6
λ_{max} (nm)	639	654	666	673	656
k_{max} ($\times 10^{-3}$ J^{-1} cm^2)	~0	~0	~0	~0	~35.87

Table 4
Relative rate constants of bleaching Ce6 in DPPC liposomes under different conditions

Conditions	Ce6
No additive	1
NaN_3 (14 mM)	0.92
SOD (0.06 mg/ml)	1.58
Catalase (0.04 mg/ml)	1.91
SOD + catalase	2.03
Mannitol (5 mM)	0.59
30 min nitrogen bubbling before irradiation	1.06
D_2O (70%)	2.72

the aqueous layer and thus cannot quench singlet oxygen in the lipid phase effectively. Due to its hydrophilicity, the majority of chlorin Ce6 will reside in the aqueous phase and thus any quenching of photobleaching by sodium azide would give a true indication of the role of singlet oxygen in the process. Consequently, it appears that singlet oxygen plays a more important role in Ce6 photobleaching in DMF than in DPPC liposomes [18]. Recombination of ionic species is favored in solvents of low dielectric constant [25]. Thus the lifetimes of radicals generated in organized media might be increased because of the large amount of water, and the radical path resulting in Ce6 photobleaching might be more prominent in liposomal media than in organic solution.

In summary, chlorins showed media-dependent bleaching behaviors, which seemed to correlate with the polarity of the microenvironment around chlorins molecules. Following incubation with DPPC liposomes, hydrophobic chlorins such as PPa, OEC, MTPC could penetrate into the lipid bilayer, where they were much more resistant to light than in DMF. This parallels the results of Handa et al. who showed that tetraphenyl porphine is much more photostable in lipid bilayers

Table 5
Densities of frontier electrons of the carbon atoms of the substituting groups in chlorins

Ce6	PPa	OEC	MTPC
$\varepsilon_{HOMO} = -7.785$ ev	$\varepsilon_{HOMO} = -7.674$ ev	$\varepsilon_{HOMO} = -7.391$ ev	$\varepsilon_{HOMO} = -7.449$ ev
C1 0.01175	**C1 0.006594**	C1 0.001797	C1 0.0003619
C2 0.05026	**C2 0.02933**	**C2 0.006662**	**C2 0.001943**
C3 0.003681	C3 0.004409	C3 0.002425	C3 0.0002043
C4 0.007510	**C4 0.007241**	**C4 0.008794**	C4 0.0002096
C5 0.001012	C5 0.002349	**C5 0.007134**	C5 0.0002392
C6 0.0001082	C6 0.0001150	C6 0.0005051	**C6 0.001481**
C7 0.00004212	C7 0.00005468	C7 0.002760	C7 0.00005180
C8 0.001649	C8 0.00003706	C8 0.00007974	C8 0.00005096
C9 0.00004420	C9 0.0002724	C9 0.0002176	C9 0.000009960
C10 0.00009674	C10 0.00001322	C10 0.0005986	C10 0.000008820
C11 0.0001048	C11 0.00005636	C11 0.00008900	C11 0.000001600
C12 0.001319	C12 0.0004352	C12 0.0001915	C12 0.00003172
C13 0.003630	C13 0.001178	C13 0.001199	C13 0.00009240
C14 0.0004159	C14 0.003333	C14 0.003432	C14 0.0001976
	C15 0.0005106	C15 0.0001661	C15 0.0001589
		C16 0.0004435	C16 0.0001821
			C17 0.00004684
			C18 0.0004676
			C19 0.0005020
			C20 0.0006999
			C21 0.0004213
			C22 0.0005602
			C23 0.00009964
			C24 0.001438

than in aqueous solvents [26] and the observation of Spikes who found that porphyrins are more resistant to photobleaching in solvents of low polarity [5]. As to hydrophilic Ce6, most of this compound tends to exist the more polar aqueous phase. Consequently, Ce6 photodegraded more significantly in liposomal media.

3.2. Structure — photobleaching relationships

Based on the above experimental results, the sequence of photobleaching efficiencies of chlorins is Ce6 > PPa > OEC > MTPC > C. It was reported that porphyrins are well known to sensitize their own photooxidation through side-chain oxidation [15]. According to a study on the mechanisms for photooxidation of protoporphyrin IX [18], sensitizers containing vinyl group are susceptible to attack by $^1O_2$ and contribute to their photooxidation. Consequently this is a possible mechanism for the photooxidation of Ce6 and PPa, both which contain vinyl groups. Since Chlorin has no side chain, the possibility of active oxygen attack on the periphery of the molecule, resulting in significant degradation, is excluded. As to OEC and MTPC, which contain ethyl groups and phenyl groups, both are subject to attack by $^1O_2$.

In order to investigate the structural reasons for photobleaching efficiencies of chlorins, we performed quantum chemical calculations on five chlorins. These studies were an extension of the work of Fukui et al. [27,28] who used frontier orbital calculations to predict the position of electrophilic attack on electron-rich centers. On the basis of that work it is possible to predict the positions of the attack in a variety of conjugated molecules and the relative reactivity of molecules. The calculated densities of frontier electrons of the substituents in Ce6, PPa, OEC and MTPC are given in Table 5.

Table 6
Densities of frontier electrons of the carbon atoms of the substituting groups in chlorin radical cations

Ce6	PPa	OEC	MTPC
$\varepsilon_{SOMO} = -7.702$ ev	$\varepsilon_{SOMO} = -8.660$ ev	$\varepsilon_{SOMO} = -8.282$ ev	$\varepsilon_{SOMO} = -8.169$ ev
C1 0.008046	C1 0.004655	C1 0.001146	C1 0.0008999
C2 0.03733	C2 0.01982	C2 0.003920	C2 0.005455
C3 0.003042	C3 0.004183	C3 0.002100	C3 0.0005941
C4 0.009390	C4 0.007621	C4 0.007658	C4 0.0004527
C5 0.001532	C5 0.002995	C5 0.008891	C5 0.0006148
C6 0.0001264	C6 0.0001430	C6 0.0006642	C6 0.004913
C7 0.00005241	C7 0.00006900	C7 0.003994	C7 0.0001848
C8 0.001773	C8 0.0001155	C8 0.0001225	C8 0.001048
C9 0.00005300	C9 0.0005836	C9 0.0001399	C9 0.00009928
C10 0.00007812	C10 0.00002724	C10 0.0002936	C10 0.0002788
C11 0.0002286	C11 0.00003898	C11 0.0002615	C11 0.0001431
C12 0.001217	C12 0.0006247	C12 0.0007707	C12 0.001194
C13 0.003165	C13 0.001028	C13 0.0008830	C13 0.0003959
C14 0.0005370	C14 0.002754	C14 0.002411	C14 0.001276
	C15 0.0005089	C15 0.0002369	C15 0.00005780
		C16 0.0006223	C16 0.0005433
			C17 0.0002806
			C18 0.001075
			C19 0.0001891
			C20 0.0003041
			C21 0.00007522
			C22 0.00009204
			C23 0.0001977
			C24 0.0001569

Based on these results, the carbon atoms of the vinyl groups in Ce6 and PPa have higher frontier electron densities and are more susceptible to $^1O_2$ attack. Moreover, the carbon atoms furthest from the center of the macrocycles have the highest reactivity (C2 in Ce6 and C2 in PPa). This is consistent with the case of photooxidation of protoporphyrin IX [18]. In addition, the methyl groups (C4) on the reduced pyrrole rings in Ce6 and PPa are active. This also indicated that Ce6 would be photooxidized more seriously than PPa, and is in accordance with the experimental results in DMF solution, where $^1O_2$ pathway is the main photobleaching process. As for OEC and MTPC, the frontier electron densities of the carbon atoms in their substituent groups are far smaller than those for the carbon atoms in vinyl groups, and the bleaching efficacy of these compounds was far lower than that of Ce6 and PPa. We also found that the position of the same substituent contributes to the reactivity of carbon atoms. C4 in OEC and C2 in MTPC are the most susceptible to $^1O_2$ attack. Ultimately, the order of the reactivity of ground-state chlorins with $^1O_2$ is Ce6 > PPa > OEC > MTPC.

As photobleaching of chlorins might be partly caused by attack of superoxide on chlorin radical cations, the frontier electron densities of chlorin radical cations were calculated and the results are presented in Table 6.

Comparing Table 5 with Table 6, it is clear that there are some differences between the positions that are susceptible to $^1O_2$ attack and radical attack. For example, the carbon atom of vinyl group near the macrocycle of Ce6 is more susceptible to $^1O_2$ attack than the methyl group on the reduced pyrrole ring. However, the latter is more susceptible to radical attack. As for PPa, the carbon atom of vinyl group near the macrocycle and the methyl group on the reduced pyrrole ring have almost equal reactivity with $^1O_2$. However, the latter is more sensitive to radical attack. C4, C5 in OEC and C2, C6 in MTPC are susceptible to both $^1O_2$ and radicals attack, but C2 in OEC and C24 in MTPC that are sensitive to $^1O_2$ but are less sensitive to radical attack, on the basis of Table 6, the sequence of the reactivity of chlorin radical cations with radical reagents is Ce6 > PPa > OEC > MTPC.

4. Conclusion

Except for chlorin C, the chlorins examined in this study were photobleached in DMF. However, only Ce6 showed obvious bleaching in liposomal media. The photobleaching efficiencies of these compounds correlated with their chemical structures. As the efficiencies and the side effects of PDT correlate with the photobleaching efficacy of sensitizers, it is necessary to take this into consideration when we screen new photosensitizers. Further work is needed to ascertain the products of sensitizer photodegradation and their photophysical, photochemical and biological properties.

Acknowledgements

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