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Properties of Chlorophyll and Derivatives in Homogeneous and Microheterogeneous Systems

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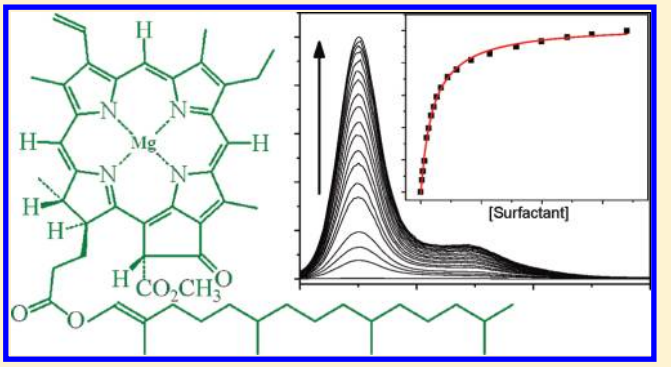
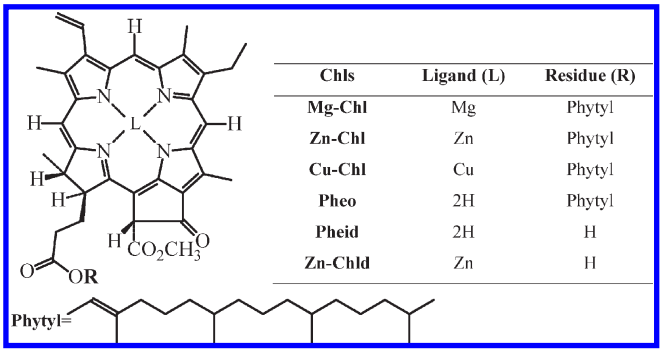
ABSTRACT: Chlorophyll (Mg-Chl) and its derivatives, zinc chlorophyll (Zn-Chl), copper chlorophyll (Cu-Chl), pheophytin (Pheo), pheophorbide (Pheid), and zinc chlorophyllide (Zn-Chld), were studied as to their acid−base equilibrium properties, hydrophobicity, stability, binding, and relative localization in neutral surfactant micellar systems. The stability order of metalochlorophyll (pH_M) in acidic medium was found to be Cu-Chl > Zn-Chld > Zn-Chl > Mg-Chl. The apparent pK_a for protonation of porphyrin ring nitrogens was around 1.0 for all derivatives. The pK_a for protonation of carboxylate phorbide was 5.9 for Pheid and 2.4 for Zn-Chld. This difference was attributed to complexation of carboxylate with zinc. The hydrophobicity of chlorophyll in relation to the ability of partitioning the cell membrane lipid layer was estimated in the octanol/water biphasic system. Pheo, a more hydrophobic molecule, presented the highest partition coefficient (K_P) in the organic phase, followed by Cu-Chl, Mg-Chl, Zn-Chl, Pheid, and Zn-Chld. The hydrophobic character was the key to relative drug location in the micellar systems. All studied derivatives interacted strongly with Tween 80 micellar systems, and particularly with P-123. For both surfactants, the order followed by binding constant (K_b) was Zn-Chld > Pheo > Cu-Chl > Mg-Chl > Zn-Chl > Pheid, while binding constants estimated for the Chl containing the phytyl group correlated with K_P. Fluorescence quenching studies have shown that phorbides are located in a less hydrophobic region than the phytyl chain-containing derivatives, which are located preferentially in a deeper micellar microenvironment. Thus, the association of the chlorophylls with specific binding sites of micellar systems is strongly modulated by the presence of phytyl chains and metal coordinated to the porphyrinic ring.



INTRODUCTION

Chlorophylls (Chl) are a class of molecules found in abundance in nature and are fundamental in the photosynthesis processes, together with other pigments like carothenoids and bacteriochlorophylls. Chl acts in photon capturing and excitation energy transfer and storage in the antennae in reaction centers (RC) in photosystems I and II.[1,2] The interaction of Chl with apoproteins changes the π-electron system, leading to variations in redox potential and spectral properties of these compounds and functional versatility as photosynthetic cofactors.[3−9]

The molecular structure of chlorophyll is a porphyrin ring with one reduced pyrrole ring (chlorine-type compound) with a Mg^{2+} ion coordinated to nitrogens and a long nonpolar phytylic chain, which enhances the hydrophobicity of the molecule as shown to chlorophyll *a* derivatives in Figure 1. The substitution of Mg^{2+} ion in the tetrapyrrole ring of chlorophyll by hydrogen atoms results in pheophytin (Pheo), which presents a more hydrophobic character than its precursor.[10] Chlorophylls complexed with the divalent cations Zn^{2+} and Cu^{2+}, known as transmetalated chlorophylls, are termed chlorophyll zinc (Zn-Chl) and copper (Cu-Chl), respectively. The Zn and Cu chlorophylls present high stability in acidic media[11] and photostability[12,13] as compared to Mg-Chl (the Chl form usually found in nature). Derivatives without the phytyl chain from free base chlorine and chlorophylls are the phorbides and



Chls	Ligand (L)	Residue (R)
Mg-Chl	Mg	Phytyl
Zn-Chl	Zn	Phytyl
Cu-Chl	Cu	Phytyl
Pheo	2H	Phytyl
Pheid	2H	H
Zn-Chld	Zn	H

Figure 1. Scheme of chlorophyll *a* structure and derivatives.

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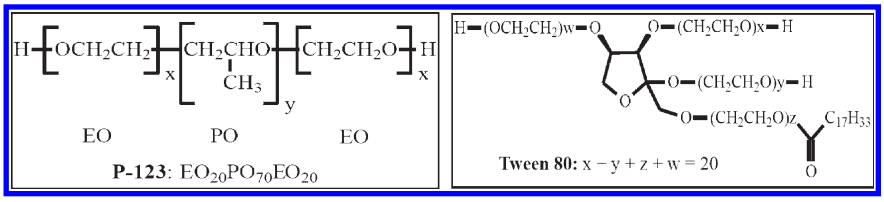

Figure 2. Structure of surfactant P-123 and Tween 80.

chlorophyllides, respectively, as evidenced by pheophorbide (Pheid) and zinc chlorophyllide (Zn-Chld) (structures presented in Figure 1).

The electronic absorption spectra of porphyrins in the visible and near UV wavelengths can be explained by the theory of the four frontier orbitals.[14] The Soret bands in the blue region arises from the $S_0 - S_n$ transitions with $n > 2$, while the Q bands in the red region, from the S_0 - S_1 and $S_0 - S_2$ to Q_y and Q_x transitions,[15,16] respectively. The chlorophyll derivatives have a high molar absorptivity coefficient (ε) in the Q bands, which, allied to other characteristics such as a long lifetime of the excited state and high singlet oxygen quantum yield (unless to CuChl),[13] makes these compounds potentially applicable as photosensitizers in photodynamic therapy (PDT).[17]

The high hydrophobicity of the chlorophylls arises from the porphyrin ring, especially for free base porphyrins; the phytyl chain causes aggregation in aqueous media and enhances the hydrophobicity. In plant cells, the phytyl chain acts as an anchor in the positioning of chlorophyll molecules to the proper orientation in the chlorophyll−protein complexes within the chloroplasts during the process of photosynthesis. Several studies have shown the importance of the phytyl chain in the interaction of Chl with hydrophobic environments and in aggregation phenomena.[18−20] The presence and type of metal coordinated to the porphyrin ring are also crucial for the conformation of these molecules in a given environment. Conformation changes drastically alter the properties of chlorophyll, such as generation and lifetime of excited states, directly affecting photochemical processes following light absorption.[21]

The complexity of natural membranes complicates the study of properties of Chl in biological tissues of animals, for instance. As the number and distribution of Chl components are highly variable, substantial study at different levels is required. Thus, model systems of simple membranes are employed, providing useful information about the properties of the molecules bound to organized systems. The micellar systems consisting of an internal hydrophobic region and a hydrophilic external aqueous solution have been used as very simple models of biological membrane interfaces.[22]

The use of surfactants is known to be effective in the stabilization of hydrophobic molecules in aqueous medium, such as photosensitizers monomerization. In clinical/pharmaceutical applications of polymeric surfactants is interesting to use neutral such as Tween 80 and polymers in block-type oxy-alkyl (Pluronics) due to their low toxicity compared to conventional surfactants. The balance of hydrophobicity of polymeric micelles is controlled by relative number of hydrophilic and hydrophobic monomers, resulting in the great versatility of these compounds, as can be noted from the number of copolymers commercially available for different uses in different areas, including drug solubilization and drug delivery.[23] Regarding the structure of the copolymer in the stabilization of hydrophobic drugs, the increase of hydrophobic blocks and the micelle molecular weight should favor drug solubilization.[24] Additionally, the incorporation of drugs that have acid−base groups in polymeric micelles should be pH dependent, once their charged forms are less stabilized within the micelle and thus may be released faster than the more hydrophobic/neutral especies.[25]

In this work, the physicochemical properties of chlorophyll *a* derivatives in homogeneous systems have been studied in terms of their acid−basic equilibrium properties, stability of metal complexes with acid treatment, and determination of the partition in the biphasic system octanol/water. Interactions of chlorophyll and its derivatives with micellar systems of neutral Tween 80 and polymeric surfactant P-123 (Figure 2) were also investigated through binding and relative localization studies.

EXPERIMENTAL METHODS

Samples: Chlorophyll Derivatives. The chlorophyll *a* derivatives Mg-Chl, Zn-Chl, Cu-Chl, Pheo and Pheid were prepared according to the procedures described in the literature. Zn-Chld was generated through metalation of Pheid in a methodology similar to that used to obtain Zn-Chl from Pheo.[26,27]

All stock solutions (10^{-3} M) were prepared by dissolving the pure chlorophyll derivative in DMSO. The stock was standardized by absorption electronic spectroscopy in acetone (UV−vis spectrophotometer Varian, model Cary 50). The molar absorptivity coefficient (ε) values were ε_{663nm} = 92 600 M^{-1} cm^{-1} for Mg-Chl; ε_{667nm} = 67 000 M^{-1} cm^{-1} for Pheo; ε_{667nm} = 56 200 M^{-1} cm^{-1} for Pheid; $\varepsilon_{656\ nm}$ = 90 000 M^{-1} cm^{-1} for Zn-Chl, ε_{656nm} = 64 000 M^{-1} cm^{-1} for Zn-Chld; and ε_{650nm} = 60 000 M^{-1} cm^{-1} for Cu-Chl.[28−30]

Fluorescence Quantum Yield (Φ_{Fl}). The Φ_{Fl} of Chl were determined in 10% water/ethanol (v/v) solutions using Mg-Chl in ethyl ether as a standard solution, Φ_{Fl} = 0.35.[31] The emission spectra were obtained with a fluorometer (Cary Eclipse, Varian). All samples presented absorbance lower than 0.05 to avoid internal filter problems. The Φ_{Fl} was calculated by eq 1.[32]

$$\Phi_{Fl} = \frac{Abs_s F_c n_p^2}{Abs_c F_s n_c^2} \Phi_P \qquad (1)$$

where *S* is the standard and *C* is Chl, *n* is the refractive index, and *F* is the emission spectra area. The excitation wavelength was fixed at 411 nm and the emission was registered in the range of 600−800 nm, samples at 25 °C.

Analysis of pH-dependence of Chl derivatives in homogeneous media: apparent constants of protonation (K_a) and demetalation pH (pH_M) investigated by electronic absorption spectroscopy.

The demetalation (pH_M) of Chl derivatives, such as Mg-Chl, Zn-Chl, and Zn-Chld, toward the Pheo or Pheid forms, as well as the subsequent protonation of the porphyrin ring's nitrogens of the free-base porphyrins and phorbide forms (pK_a), was investigated in homogeneous media comprised of 10% water/ethanol

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(v/v) solutions. Further protonation of the carboxylic group of phorbides was also assessed.

Their pH_M and pK_a values were determined by monitoring the electronic absorption spectra of Chl (5×10^{-6} M) as a function of the medium pH. The studies were performed in 10% water/ethanol solutions (v/v) in order to maintain the Chl in a monomeric state.[26] In this way, the pH of 10% water/ethanol (v/v) solutions in a quartz cuvette containing the Chl derivative was adjusted with the addition of small aliquots (μL) of acid stock solution (6 M HCl) at 30.0 °C. The titration UV−vis spectra to Chl were obtained after complete demetalation, and the pK_a and pH_M values were evaluated through statistical treatment of experimental data using principal component analysis (PCA), imbrie Q-mode analysis, and the K matrix method.[33]

Determination of Octanol/Water Partition Coefficient. To a biphasic mixture containing octanol/water at 50% (v/v) was added the Chl (2×10^{-6} M), and after intense stirring and 48-h rest in the dark, the Chl concentration in both phases was evaluated by UV−vis. The partition coefficient of the octanol phase (K_P) was calculated by

$$K_P = [\mathrm{Chl}]_0/[\mathrm{Chl}]_w \quad (2)$$

where $[\mathrm{Chl}]_o$ and $[\mathrm{Chl}]_w$ are the molar concentrations of Chl in octanol and water, respectively.

The molar absorptivity coefficient (ε) values in $Q_{y,0-0}$ band used were 55 700 M^{-1} cm^{-1} for Mg-Chl; 26 500 M^{-1} cm^{-1} for Pheo; 30 100 M^{-1} cm^{-1} for Pheid; 62 100 M^{-1} cm^{-1} for Zn-Chl, 57 100 M^{-1} cm^{-1} for Zn-Chld; and 26 200 M^{-1} cm^{-1} for Cu-Chl in water. The ε values in octanol used were of 95 700 M^{-1} cm^{-1} for Mg-Chl; 77 600 M^{-1} cm^{-1} for Pheo; 88 700 M^{-1} cm^{-1} for Pheid; 93 000 M^{-1} cm^{-1} for Zn-Chl, 87 500 M^{-1} cm^{-1} for Zn-Chld; and 63 000 M^{-1} cm^{-1} for Cu-Chl.

Interaction of Chl Derivatives with Micellar Microenvironments: Fluorescence Studies. The surfactant Tween 80 [Synth, MM = 1310 g mol^{-1}, critical micelle concentration (cmc) = 1.2×10^{-5} M] and the pluronic P-123 [Aldrich, MM = 5800 g mol^{-1}, cmc = 4.4×10^{-6} M][23] were used in the binding constant experiments and the Stern-volmer quenching studies with the Chl. Static fluorescence emission of Chl at 5×10^{-7} M was monitored in a Varian-Cary Eclipse spectrofluorometer with excitation wavelength (λ_{exc}) of 411 nm. All experiments were performed at 30.0 °C.

Binding Constants (K_b) of Chl with Polymeric Micelles. The binding constants (K_b) of Chl with micelles were evaluated by the fluorescence emission spectra of Chl (5×10^{-7} M) acquired in the presence of either P-123 or Tween 80 solutions. The amount of Chl was reduced by 10 times in comparison with UV−vis experiments in order to avoid the inner filter effect and to reduce aggregation effect. Aliquots of surfactant were added using a 2% (w/v) stock solution directly to a cuvette containing Chl in DMSO ([DMSO] < 0.1% (v/v)) in aqueous solution. The experimental data were theoretically fitted using eq 3.[34]

$$F = F_0 + (F_f - F_0)/((1/K_b([S] - \mathrm{cmc})^N) + 1) \quad (3)$$

where F is the fluorescence emission intensity of Chl, F_f is the emission of Chl bound to the polymeric micelle, F_0 is the emission of unbound Chl, K_b is the binding constant; [S]: surfactant concentration, and N is the number of surfactant molecules per molecule of Chl.

Quenching Fluorescence Studies. Additionally, quenching fluorescence experiments were performed with titration of Chl solution (5×10^{-7} M) in the presence of micellar systems P-123 (3.15×10^{-4} M) and Tween 80 (1.5×10^{-3} M), using I^- from a 3 M KI stock solution as a water-soluble quencher. Stern−Volmer constants (K_{SV}) were calculated through[35]

$$F_0/F = 1 + K_{SV}[I^-] \quad (4)$$

where F_0 and F are the fluorescence emission of pure Chl in solution and bound to surfactants, respectively, and $[I^-]$ is the concentration of the iodide (quencher). The concentrations of surfactants were kept above their cmc.

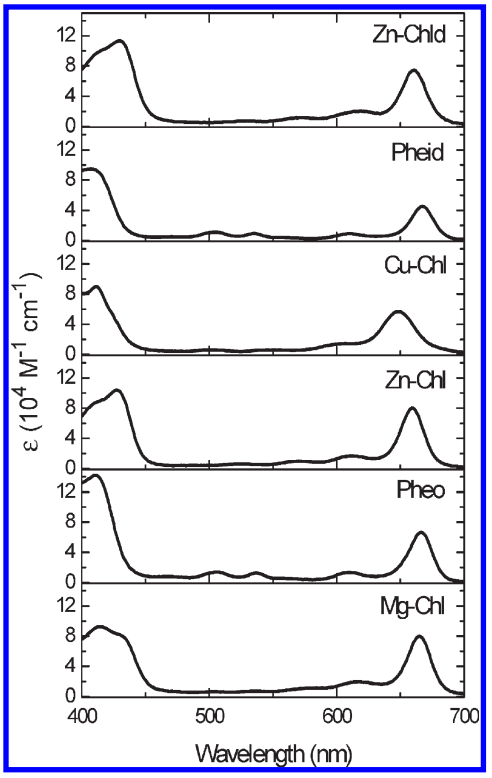

Figure 3. Electronic spectra of Chls in 10% water/ethanol, 30.0 °C.

RESULTS

Spectroscopic Properties of Chlorophyll Derivatives in Homogeneous System. The electronic absorption spectra of Chl in 10% water/ethanol (v/v) solutions are presented in Figure 3. Two main characteristic absorption bands of chlorines can be noted, namely Soret (also known as B-band, at around 350−400 nm) and Q-band (most prominent peak at c.a. 600−660 nm). However, in the free-base compounds, Pheo and Pheid, additional weaker bands are observed in the range of 500−650 nm, arising from the asymmetry of the porphyrin ring.[36] Figure 3 shows that the Q-Bands of Chl complexed with Zn^{2+} and Mg^{2+} present relatively higher absorption intensities than those observed in free base forms and metalated with Cu^{2+}. On the other hand, similar to that observed in organic media,[19] the electronic spectra are affected only slightly by the phytyl group, as observed in Pheo and Pheid, as well as in Zn-Chl and Zn-Chld, keeping the spectral profile and with changes in molar absorptivity. The wavelengths of maximum absorption (λ_{abs}), molar absorption coefficients (ε), and fluorescence emission (λ_{em}) from Chl spectra are shown in Table 1.

Table 1. Wavelengths of Maximum Absorption (λ_{abs}), Molar Absorption Coefficients (ε), Fluorescence Emission (λ_{em}), and Fluorescence Quantum Yield (Φ_{Fl}) of Chl Derivatives in 10% Water/Ethanol (v/v), at 30.0 °C

	Soret		$Q_{y,0-0}$		Φ_{Fl}	
Chl	λ_{abs} (nm)	ε (10^3 M^{-1} cm^{-1})	λ_{abs} (nm)	ε (10^3 M^{-1} cm^{-1})	λ_{em} (nm)[a]	(10^{-3})
Mg-Chl	430	83.1	665	79.4	667	0.5
Zn-Chl	428	103	659	80.0	663	2.1
Zn-Chld	429	11.5	660	74.2	665	16
Cu-Chl	412	90.0	650	56.0	668	~0
Pheo	411	142	667	66.7	671	6.0
Pheid	411	93.2	667	45.2	673	96

[a] λ_{exc} = 411 nm.

Figure 4A gives the electronic absorption spectra obtained during the Chl demetalation by acid adition in 10% water/ethanol solution, which show that the spectral properties of Chl (absorption and λ_{max}) are pH-dependent. Upon acidification of the media, the demetalation effect of Mg-Chl was detected, and new species with distinct spectral features were formed. For instance, two main Q-bands, at λ_{max} = 665 ($Q_{y,0-0}$) and 615 nm ($Q_{y,0-1}$), were observed initially in the Mg-Chl. Upon the reaction of demetalation and the subsequent formation of free base Pheo, the loss of symmetry of the porphyrinic ring leads to the appearance of four distinct new absorption bands.[36] The $Q_{y,0-0}$ band intensity decreased in comparison with that of Mg-Chl, while a slight red shift to 667 nm was observed. Furthermore, Pheo presented a higher molar absorption coefficient with a bathochromic shift in the Soret band (Table 1 and Figure 3).

Figure 4B presents the UV−vis spectra of acid titration of free base form Pheo. The spectral changes observed with Pheo protonation are again a consequence of changes in the porphyrin ring symmetry.[36] In this case, a hypsochromic shift and decrease of the intensity of $Q_{y,0-0}$ band were observed in the protonated form of $Pheo^{+2}$, as compared to free base form of Pheo. The vibronic components $Q_{y,0-1}$ and $Q_{x,0-0}$ were also altered in position and intensity. For instance, the $Q_{x,0-1}$ band at λ_{max} = 509 nm disappeared (to the vibronic component of the second transition polarized along the *x* axis), and a significant increase of the absorption intensity of the Soret band was detected.[37] Similar changes in the spectra profile were also observed upon the acid titration of the Zn-Chl.

In addition, the dicationic species ($Pheo^{2+}$ and $Pheid^{2+}$) possess practically identical electronic spectra, independently of the presence or absence of the phytyl chain in their structure.[19] Besides, the protonation of the propionate group in both phorbide compounds lead to a very slight change in their UV−vis spectra, as explored in chemometric studies (Figures 5 and 6).

Chlorin Acid/Basic Equilibria in Homogeneous Media: pK_a and Demetalation pH (pH_M) Determination. As observed in Figure 4, the compounds Mg-Chl, Pheo, and $Pheo^{2+}$ presented a remarkable spectral difference that allowed the determination of pH_M (Figure 4A), as well as of the acid equilibria constant (pK_{a1}) of the porphyrinic ring (Figure 4B).

The electronic absorption spectra of pure species of the Chl were evaluated by chemometry (K Matrix method) and are exhibited in Figure 5A. The schematic representations of the chemical equilibria of the molecules involved in the acidification steps are presented from right to left as an insert in the same Figure 5A. In Figure 5B, the fractions calculated of each species of Mg-Chl as a function of pH are shown.

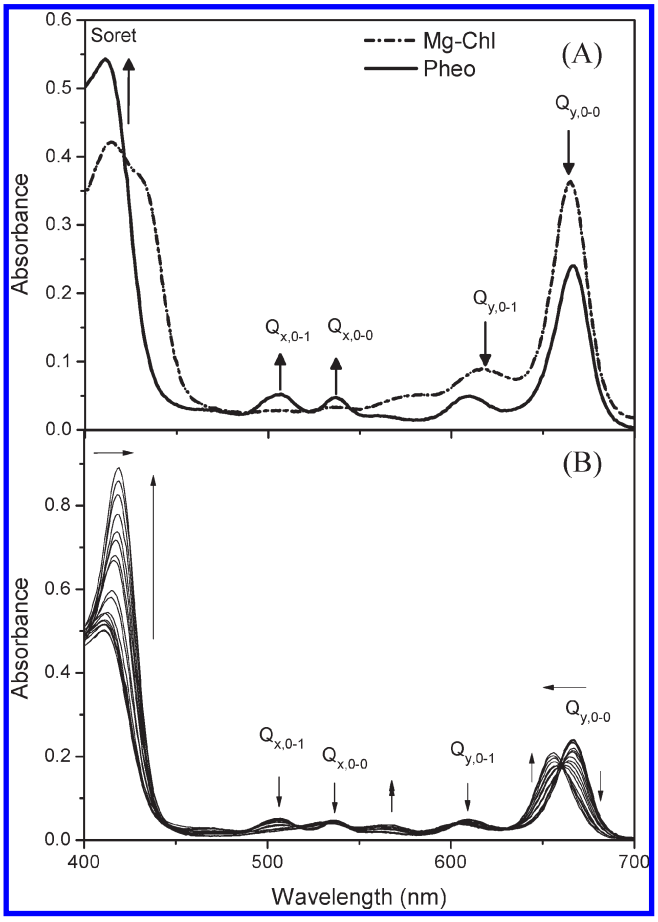

Figure 4. Electronic absorption spectra in 10% water/ethanol solution showing (A) the demetalation of chlorophyll (Mg-Chl) and formation of Pheo and (B) the protonation of Pheo producing $Pheo^{2+}$. The direction of the arrows indicates the changes in spectra with acidification of the medium (pH 9.0 down to 0.5 at 30.0 °C).

For instance, in Figure 5B, the predominance of the metalated Mg-Chl can be observed, from basic pH values down to the lowest reactional pH 3.5, at which the metal center was replaced by protons. In the pH range of 3.5 down to 1.0, the Pheo form prevailed, while at pH below 1.0, mostly the dicationic ($Pheo^{2+}$) species was formed.[38] The same behavior was observed for Zn-Chl with the lowest pH_M found of 2.3. The values of pH_M and pK_a estimated for all derivatives are shown in Table 2.

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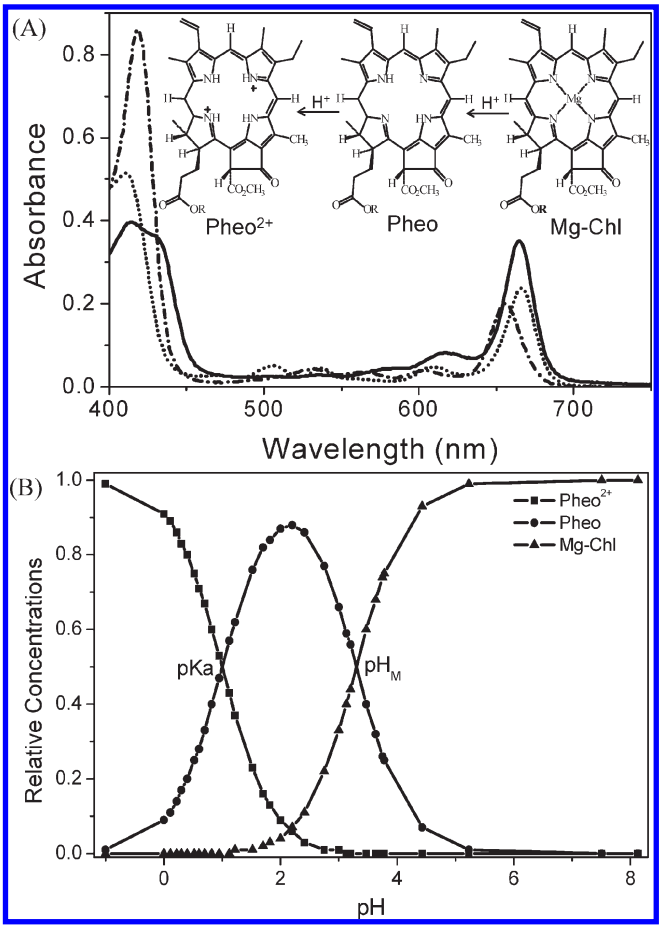

Figure 5. (A) Comparison of the absorption spectra of Mg-Chl, Pheo, and $Pheo^{2+}$ and schematic representation of the process of demetalation of chlorophyll and protonation of Pheo. Solid line, Mg-Chl; dotted line, Pheo; dashed dotted line, $Pheo^{2+}$. (B) Relative concentrations of species versus pH in 10% water/ethanol solution; 30.0 °C.

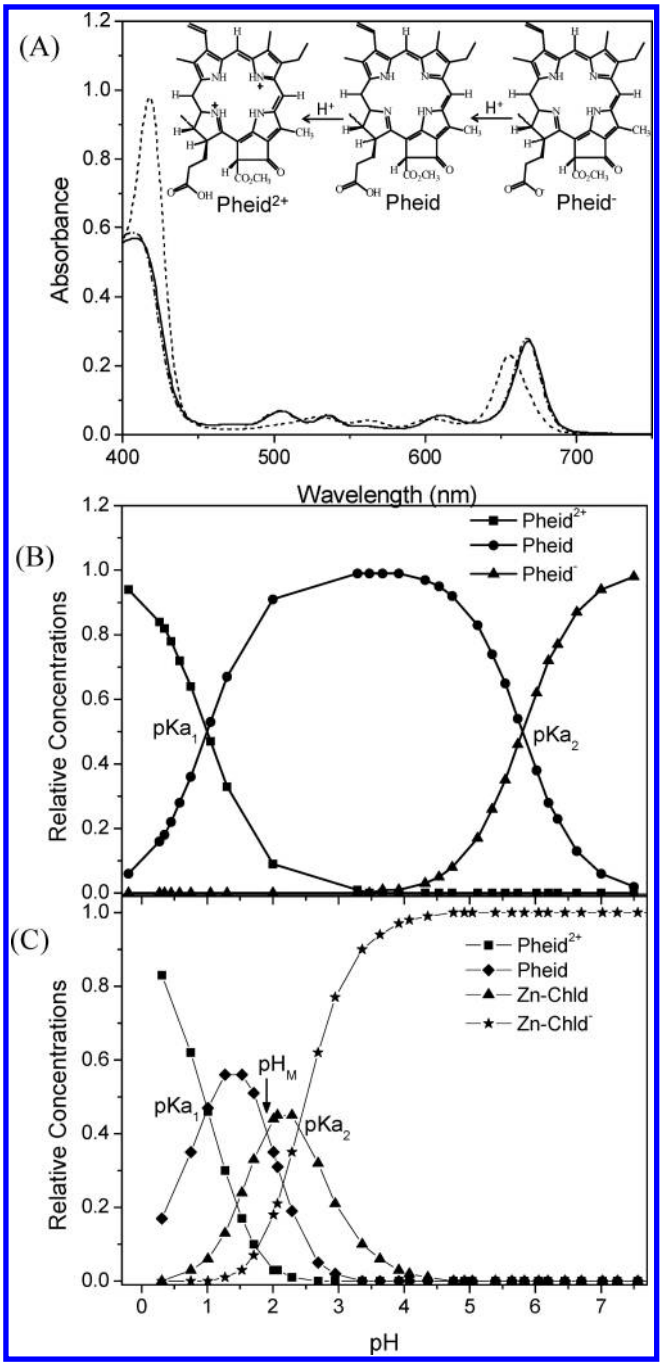

Figure 6. (A) Absorption spectra of species for the acid−base equilibria of Pheid with schematic representation of protonation of the carboxylic group and the nitrogens of the porphyrin ring in 10% water/ethanol solution; 30.0 °C. Solid line, $Pheid^{-}$; dotted line, Pheid; dashed dotted line, $Pheid^{2+}$ (B) Relative concentration of protolytic species of Pheid as a function of pH at 30.0 °C. (C) Relative concentration of protolytic species of Zn-Chld as a function of pH.

For instance, the electronic absorption spectra and schematic representation of pure phorbide forms involved in acid−base equilibria of Pheid, which possesses a propionic acid group bound to C-17, are described in Figure 6A for 10% water/ethanol solutions. The anionic form $Pheid^{-}$ and its uncharged protonated form Pheid presented almost identical absorption spectra, whereas the equilibrium was pK_{a2} 5.9, and again the pK_{a1} was around 1.0 (Figure 6B).

Nevertheless, from Figure 6C, significant changes were observed in the Zn-Chld system, as well as a further distribution of species related to the metalated forms is evident. The presence of zinc shifted the pK_{a2} value to a pH region as low as 2.4, in contrast to 5.9, as was observed for Pheid (Table 2). Moreover, the pH_M estimated for Zn-Chld was 1.9, whereas the value obtained for Zn-Chl was 2.3.

The pK_{a1} values corresponded to equilibria involving only the protonation of the porphyrin ring was around 1.0 for all systems studied, except for the system containing Cu-Chl, stable complex in acidic medium.

Determination of Octanol/Water Partition Coefficient. Ensuring that the metalated structure and protolytic species remained unaltered and assuming that equilibria these systems do not change, the studies of the properties of Chl derivatives were performed in biphasic octanol/water and microheterogeneous systems (micellar solutions) and of interaction with the model systems at pH ~6.0, above their pH_M and pK_{a1} values.

The values obtained for log K_P for all Chl were greater than 0. Pheo presented the highest partition coefficient for the organic phase (K_P = 326). Regarding the Chl with the phytyl group, the K_P observed for the compounds followed the order Pheo > Cu-Chl > Mg-Chl > Zn-Chl (Table 3), with the partition values

Table 2. Values of pKa and pH_M Chlorophylls in 10% Water/Ethanol, at 30.0 °C

Chl	pK_{a1}[a]	pH_M[b]	pK_{a2}[c]
Mg-Chl	1.0	3.5	
Cu-Chl			
Zn-Chl	1.1	2.3	
Zn-Chld	1.0	1.9	2.4
Pheid	1.0		5.9
Pheo	1.1		

[a] pK_{a1}: logarithm of acid constant of the nitrogen of the porphyrin ring. [b] pH_M: logarithm of hydrogen ion concentration necessary for the demetalation of the metal-porphyrin complex. [c] pK_{a2}: logarithm of the acid constant of the carboxylic group (propionic acid residue).

Table 3. Octanol/Water Partition Coefficient of Chlorophylls

Chl	K_P	log K_P
Mg-Chl	275	2.44
Zn-Chl	247	2.39
Cu-Chl	283	2.45
Zn-Chld	32	1.50
Pheo	326	2.51
Pheid	209	2.32

being somewhat similar among the chlorophylls. On the other hand, the phorbide derivatives presented smaller K_P values of 209 for Pheid, and 32 for Zn-Chld.

Binding of Chlorophyll Derivatives in Micellar Systems: Fluorescence Studies. The fluorescence emission spectra of Pheid (5×10^{-7} M) after adding aqueous P-123 or Tween 80 solutions are illustrated in Figure 7, panels A and B, respectively. The maximum emission intensities at λ_{max} = 675 nm as a function of surfactant concentration, presented as inserts in Figure 7, panels A and B, allowed for determining K_b.

It was observed that the emission intensity of Pheid in aqueous media was very small before the titration with surfactants (Figure 7), as observed for all the other Chl substrates, except for Zn-Chld, which is more soluble in water. Concurrently, an increase in the emission intensity was observed for all Chl with surfactant addition.

The K_b values were estimated through fitting of experimental data (inserts in Figure 7) using eq 3, shown in Table 4. Significant values of the order of 10^4 were obtained for the investigated derivatives, which is evidence of the meaningful interaction between Chl and the surfactants. Furthermore, the degree of association of Chl to the P-123 micellar system was higher than that of Tween 80.

The results (Table 4) showed that Zn-Chld presented the highest K_b value in both surfactants, followed by the Pheo, Cu-Chl, Mg-Chl, Zn-Chl, and finally Pheid.

The relative location of Chl in P-123 and Tween 80 micellar system was accessed by fluorescence quenching experiments using iodide anion (I^-) as a water-soluble quencher; the Stern−Volmer plots are presented in Figure 8. Figure 8A shows that in the P-123 system Pheid and Zn-Chld were accessed by the quencher, which was evidence of a more superficial location for these substrates, thus being more exposed to I^-. This effect was also observed in micelles of Tween 80 (Figure 8B). However, in

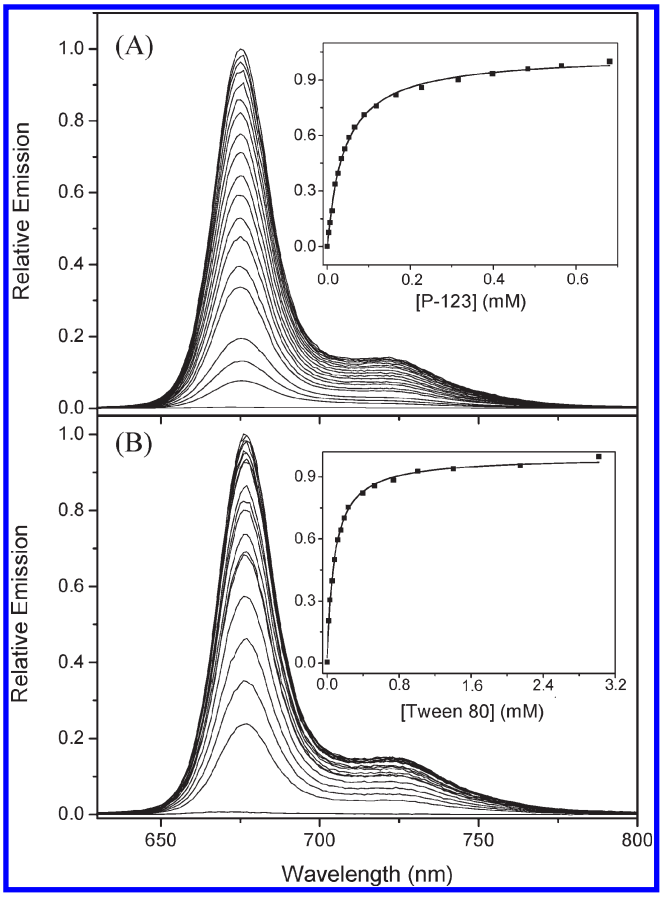

Figure 7. Fluorescence emission spectra of Pheid 5×10^{-7} M, with addition of (A) P-123 and (B) Tween 80. The inserts are related to relative emission intensities at λ_{max} = 675 nm versus concentrations of surfactants which has been fitted by eq 3.

Table 4. K_b and K_{SV} of the Chlorophylls in Micellar Systems of P-123 and Tween 80, at 30.0 °C

	P-123		Tween 80	
Chl	K_b (10^3 M^{-1})[a]	K_{SV} (M^{-1})	K_b (10^3 M^{-1})[a]	K_{SV} (M^{-1})
Mg-Chl	36.7 ± 3.3	0	22.5 ± 1.4	0.38 ± 0.01
Pheo	51.0 ± 1.3	0	41.5 ± 3.3	0.39 ± 0.01
Zn-Chl	28.6 ± 1.1	0	19.7 ± 1.1	0.57 ± 0.02
Cu-Chl	37.3 ± 1.4	0	25.9 ± 1.2	0.40 ± 0.05
Pheid	24.1 ± 0.1	0.80 ± 0.01	12.8 ± 0.4	1.10 ± 0.01
Zn-Chld	58.5 ± 3.1	0.48 ± 0.01	41.8 ± 3.1	0.84 ± 0.02

[a] N evaluated equal 1.[34]

Tween 80 micelles, all Chl fluorescence emission was quenched to some extent. Table 4 shows that fluorescence quenching efficiency in Tween 80 follows the order Pheid > Zn-Chld > Zn-Chl > Cu-Chl ~Pheo ~ Mg-Chl.

DISCUSSION

Spectroscopic Properties of Chlorophyll Derivatives. Particularly for chlorines, the high ε and bathochromic shift of the Q-band are suitable characteristics for PDT.[39] The experimental changes observed in the ε and λ_{abs} of these absorption bands for

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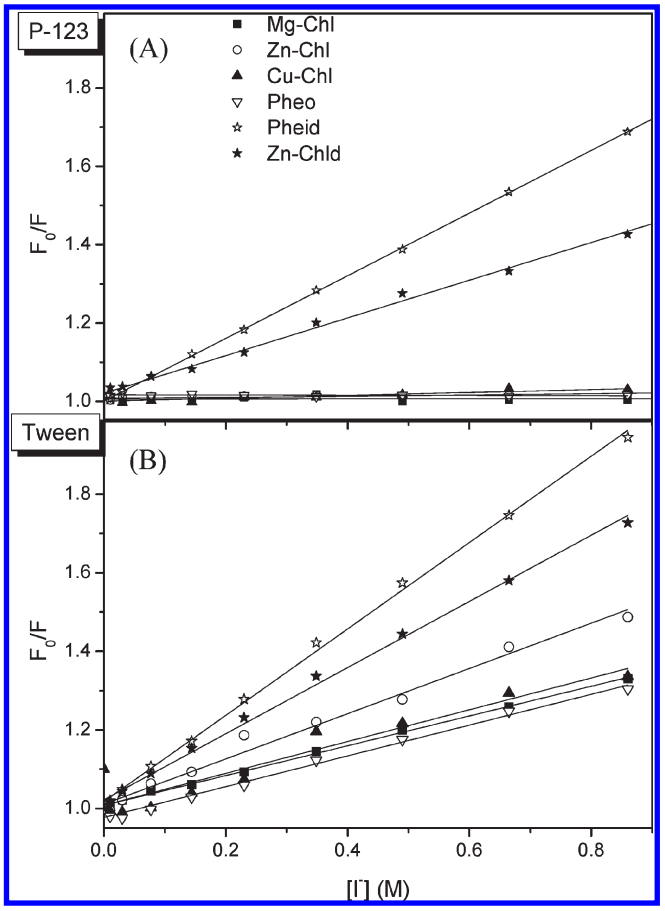

Figure 8. Stern−Volmer plots to chlorophyll derivatives (5×10^{-7} M) with $[I^-]$ in (A) P-123 (3.15×10^{-4} M) and (B) Tween 80 (1.5×10^{-3} M); 30 °C.

Chl in 10% (v/v) water/ethanol were convenient for understanding their chemical pH-dependence and photochemical properties.[40]

Concerning the spectral properties of Chl, the phytyl chain residue affects slightly the chromophoric portion of the molecule,[19] which explains the spectral similarity for substrate Pheo (phytyl chain presence) with Pheid (chain absence), also observed for Zn-Chl and Zn-Chld. Similarly, this fact justifies the spectral similarity exhibited by the forms $Pheid^-$ and Pheid, $Zn\text{-}Chld^-$ and Zn-Chld that correspond to pK_{a2} equilibria. The emission properties of these derivatives are similar in shape, while the additional shoulder observed around 720 nm has been attributed to vibrational transitions.[40]

Unless to Cu-Chl, the acidification of chlorophyll solutions results in metal-loss compounds toward Pheo and Pheid forms. The chlorophylls with different metallic centers showed a slight bathochromic shift of the Q_y-band, as compared to the spectra of Pheo and Pheid (Table 1). These changes have been associated with the stability of the complex/ligand formed. In fact, Williams observed a bathochromic shift in a variety of metalated porphyrins in agreement with the decreasing stability of the complex,[41] which is also related to the electronic affinity of the metals, i.e., the reduction of the electronic conjugation of the ring.[42] Metalation of the free base porphyrin causes meaningful changes in its energy levels. In most cases, the porphyrinic structure can change its planar configuration dependent on metal/ligand type and coordination state of metallic center, leading to changes in molecular symmetry from D_{2h} in symmetric system, due to the two hydrogens in opposite positions located in the ring center, to D_{4h}, in which all the nitrogen atoms are equivalent.

Under subsequent protonation of these two nitrogens of tetrapyrrole ring, significant modifications of the shape and intensity of the Soret Band are noted, as well as of the number of Q bands displayed. These spectral modifications are similar to that observed when Pheo and Pheid are metalated into Chl (is the same as that observed when the free base porphyrins are submitted to protonation or metalation).[36] These changes affect the π-electron conjugation due to the protonation of nitrogen, leading to molecular symmetry modifications in the porphyrinic ring.

Chlorin Acid/Basic Equilibria in Homogeneous Media. Understanding the influence of the pH on physicochemical properties of chlorophylls is important, as this may have repercussions on their stability, molecular structure, and net charge state.[37] Both pK_{a1} and pH_M are easy to determine due to the meaningful spectral changes observed in the involved species. However, the determination of the pK_{a2} in phorbide derivatives is difficult due to the spectral similarities, as previously discussed.

It is interesting to note the close values of pK_{a1} and pH_M of 1.1 and 2.3 for Zn-Chl and 1.0 and 1.9 for Zn-Chld, respectively. In these cases, traditional analytical treatments do not allow the unequivocal determination of pK_a because of band superposition and too close proximity of values.[43] However, the chemometry resulted in reliable values.[44,45]

The highest pH_M value found for Mg-Chl demonstrates its susceptibility to degradation in acid media, leading to Pheo formation. The value of pH_M of Zn-Chl (and Zn-Chld) was significantly lower than that of Mg-Chl, confirming its higher degree of stability.[41,42] Indeed, the sequence of stability of metaloporphyrins has been evaluated as $Cu^{2+} > Zn^{2+} > Mg^{2+}$. It follows the same tendency of the Irving-Williams series, which is related to hard−soft/acid−base behavior of metal ion center,[46] which agrees with our results (Table 2). The pH_M of Cu-Chl was not determined, because in extreme acid conditions the chlorophyll structure degradation was observed.[11]

Furthermore, a dramatic decrease of pK_{a2} to 2.4 associated with the carboxylate group of Zn-Chld was observed, in comparison with the pK_{a2} of 5.9 determined for Pheid (Table 2). This meaningful difference observed between pK_{a2} Zn-Chld and Pheid can been attributed to a complex formed between the metal-porphyrin ring (electron acceptor characteristics) and the propionate group (electron donor). The propionate group presents two oxygens, which favor its complexation. In fact, the Zn coordination to the carboxylate justifies the pK_{a2} shift observed toward lower values, as the electron density in the oxygens diminished, its acidic character increased. Even after the protonation leading to the carboxylic form, one of the oxygens can remain coordinated to Zn. This interaction is weak and can also explain the smallest pH_M of Zn-Chld. This derivative presented a greater stability in acid media than its precursor Zn-Chl. The influence of the propionate group on C-17 in the axial coordination of Chl was studied by Fiedor,[2] who has emphasized that this group is a strong chelant agent. Usually, this kind of complexation is found in the coordination of amino acids to Zn atoms in enzymatic systems.[47]

Finally, the pK_{a1} values related to the nitrogens of the porphyin ring protonation of free metal Pheo or Pheid compounds in their corresponding dicationic forms were around 1.0 for all systems. The values were shown to be independent of the residue substituent.

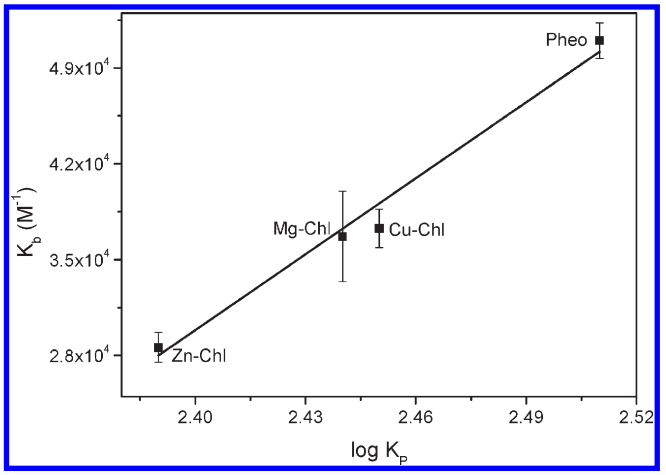

Figure 9. Relationship between binding constant (K_b) and octanol/water partition coefficient (K_P) for the CD containing phytyl chain.

Octanol/Water Partition Coefficient. In the studies of partition coefficient constants (K_P) of Chl in the octanol/water biphasic system, log K_P values were greater than 0 for all derivatives, evidencing a preferential partitioning toward to the octanol phase. This fact indicates the primary tendency of Chl to incorporate into the hydrophobic microenvironment of biomembrane interfaces or membrane bilayers.[48]

The order estimated for K_P for the derivatives with a phytyl chain was: Pheo > Cu-Chl > Mg-Chl > Zn-Chl, with similar values for the chlorophylls (log $K_P \approx 2.4$). The tendency of Pheo molecules to be partitioned into the organic phase is justified by its greater hydrophobicity in comparison with other species, such as metal-complexed ones or those without the phytyl chain derivatives. The smallest values of K_P obtained for the phorbide species might be due to the replacement of the phytyl chain by the propionate group (negative charge), a more water-friendly substituent.

The obtained results show the importance of the hydrophobic phytyl chain of such compounds in the partitioning within hydrophobic microenvironments which should be responsible for the primary interaction of chlorophyll-proteins complexes in photosynthetic organisms.[19]

Binding and Location of Chlorophyll Derivatives in Micellar Systems. The increase in fluorescence emission of Chl as a function of surfactant concentration is associated with the presence of Chl monomers bound to micelles. All derivatives presented the K_b values in P-123 micelles higher than in Tween 80. The studies evidenced the relevance of the phytyl chain on Chl structure partitioning for the octanol phase and on Chl binding to the micelles. The values of K_b for the polymeric micelles of Tween 80 and P-123 showed that substrates containing the phytyl chain followed the same sequence of hydrophobicity verified through partition coefficient (K_P) studies. The good correlation between K_b and K_P is shown through the linear relationship, as illustrated in Figure 9. It is worth mentioning that this relationship was not obeyed for the phorbide substrates. Therefore, the differences observed in K_b values are due to the hydrophobic effect, which is further modulated by the metal coordinated to the porphyrin ring in the compounds presenting the phytyl chain.

The influence of the metal on the hydrophobicity scale of phytyl chain-containing Chl might be explained by the ability of coordination of water molecules to the metal. For Cu-Chl, the cupper atom is tetracoordinated in planar geometry (d^9 complex), as well as the free base porphyrins. Therefore, the Cu^{2+} metal usually does not provide a site for axial ligand coordination, such as water molecules.[49] The large K_P value observed for Cu-Chl reflects this low water affinity.

On the other hand, the pentacoordination prevails for Chl complexes with Mg and Zn that present d^0 and d^{10} configurations, respectively. The pentacoordinated complexes provide a square base pyramidal geometry; however, hexacoordinated complexes of Mg-Chl are also frequent.[30] These complexes permit higher axial interaction/solvation by water ligands. Another factor that must be considered is the size of the metals. Zn^{2+} has a greater atomic diameter than Mg^{2+}, making the first metal leave the porphyrin plane. As a consequence, the electronegative character of Zn-Chl is enhanced, leading to high solvation by water molecules.[50,51] These effects explain the lowest K_P and K_b of Zn-Chl among the Chl derivatives presented in Figure 9 and Tables 3 and 4, respectively.

Furthermore, the linear relationship between K_b and K_P obtained for phytyl-bound substrates contrasts with the results obtained for phorbides derivatives. One of the reasons may be the existence of distinct binding sites inside the micelle microenvironments.[52] Although it is expected that the Chl with phytyl chain is located deeper within the micellar core due to the hydrophobic microenvironment, the Pheid probably is located in a less hydrophobic interfacial region of the micelle. However, the other phorbide compound, Zn-Chld, shows simultaneously the highest value of K_b and the lowest value of partition coefficient K_P. These results probably came from the already mentioned intramolecular complex formation between the Zn^{2+} and the propionate substituent.

Fluorescence quenching studies employing a hydrophilic iodide quencher, in which the substrate located near the aqueous interface is most sensitive, have confirmed the relative localization of Chl in the micelle. Only Pheid and Zn-Chld, were accessed by the quencher (I^-) in the P-123 micelle system due to their greater exposure to the aqueous micellar interface. The quenching of Pheid fluorescence by [I^-] ($K_{SV} = 0.80\ M^{-1}$) was stronger than that observed for Zn-Chld ($K_{SV} = 0.48\ M^{-1}$). All other derivatives seem to be located deeper in the hydrophobic core of the P-123 micelle.

In the case of Tween 80, the fluorescence emission of all Chl was affected due to a greater accessibility of the quencher to the substrate, attributed to the small size of the micelle and its inability to protect large substrates within. Once again, the location of Pheid in the aqueous inner-region of the micelle was demonstrated by its highest K_{SV} values in the micelles, in agreement with its lowest K_b in Tween 80 and P-123 micelles, as well as low hydrophobic K_P parameter. The differences between Pheo and Pheid regarding their association and localization in micelles are explained by the presence of the phytyl chain associated with the propionate negative charge of Pheid. The same effect was followed to Zn-Chld, however in a lesser extension. Both substrates, Pheid and Zn-Chld, exhibited the lowest partition coefficient K_P for the organic phase. The high K_{SV} value of Zn-Chld agrees with the lowest K_P, confirming the location of this substrate in a less hydrophobic region of the P-123 micelle. However, it does not justify the highest binding constant ($K_b = 58.5 \times 10^3\ M^{-1}$) for micelles of P-123. Apparently, Zn-Chld, which forms the intramolecular complex, firmly occupies the more hydrophilic poly(ethylene oxide) interfacial region of the P-123 micelles stabilized by hydrogen bonding interactions.

Hence, all results clearly evidenced that Pheo must be located preferentially in a deeper hydrophobic region of the micelle, carried by its pronounced hydrophobicity ($K_P = 326$); this effect

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was confirmed by high K_b values associated with the lowest K_{SV} among all derivatives. Despite lesser intensity, these results were followed by Cu-Chl and Mg-Chl substrates, whose locations are similar to that of Pheo in both studied micelles and also modulated by their hydrophobicity (represented by K_P). In addition, the lowest K_P and K_b exhibited by Zn-Chl among all the investigated metalated chlorophyll containing phytyl-chain are fully consistent with the highest values of K_{SV} (in Tween 80, for instance). These results once again can be justified by their hydrophobic character.

In fact, all results involving chlorophyll substrates that present the phythyl chain can be easily explained by their hydrophobic parameter. In these cases, probably the porphyrin ring is directed toward to the aqueous interface, while the phythyl chain is maintained within the micelle internal core.

CONCLUSIONS

Regarding the demetalation of Chl, the greater stability of Zn-Chl in acidic media in comparison to Mg-Chl was confirmed with pH studies employing chemometric methods. The pK_a found for the Pheid carboxylic group was 5.9, whereas a value of 2.4 was found for Zn-Chld, resulting from complexation of this group with the metallic center, which should also explain the weaker tendency of Zn-Chld demetalation in comparison to Zn-Chl. Besides, the apparent pK_a of nitrogens from the porphyrinic ring to their respective free base forms Pheo and Pheid was not affected, resulting in the same value of 1.0.

The partition coefficients in octanol/water biphasic systems have shown that chlorophylls preferably interact with hydrophobic moieties. The descending order of partition coefficient values was Pheo, Cu-Chl, Mg-Chl, Zn-Chl, Pheid, and Zn-Chld, according to the degree of hydrophobicity. Hydrophobicity was the key to the relative drug location in the micellar systems. All studied derivatives interacted strongly with Tween 80 and mainly with P-123 micellar systems. The role of the metallic center in Chl with bound phytyl-chain was evidenced through correlation between K_b and K_P values and explained by the ability of water coordination to the metals. For both surfactants, the order followed by K_b was Zn-Chld > Pheo > Cu-Chl > Mg-Chl > Zn-Chl > Pheid. Fluorescence quenching studies have shown that the phorbide derivatives are located in a less hydrophobic interfacial region than the phytyl-bound chain derivatives, which are located preferentially in a deeper hydrophobic micellar microenvironment. Thus, the association of the Chl with specific binding sites of micellar systems is strongly modulated by the presence of the phytyl chain and the metal coordinated to the porphyrinic ring.

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