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Photophysical properties of pheophorbide a in solution and in model membrane systems†

BEATE RÖDER,* TH. HANKE, ST. OELCKERS, ST. HACKBARTH AND CH. SYMIETZ

Humboldt-Universität zu Berlin, Institut für Physik, AG Photobiophysik, Invalidenstrasse 110, D-10115 Berlin, Germany

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ABSTRACT: Pheophorbide a (Pheo) is a well-known hydrophobic photosensitizer used for photodynamic treatment of various diseases. The influence of the surroundings on the electronic properties of photosensitizers mainly accumulating in membrane structures is of relevance for their photoactivity. In this paper the current knowledge about the electronic properties of Pheo in different microheterogeneous environments is summarized and new findings about its incorparation in different model membranes are discussed. Copyright © 2000 John Wiley & Sons, Ltd.

KEYWORDS: photosensitization; PDT; photophysics; singlet molecular oxygen

INTRODUCTION

Pheophorbide a (Fig. 1) represents a decomposition product of chlorophyll a. Its photosensitizing activity *in vitro* and *in vivo* is well known and has been under study for more then 15 years.

Moreover, its electronic properties, especially the high singlet oxygen quantum yield and the fast retention from tissue, favour this tetrapyrrole for use as a photosensitizing drug in photodynamic therapy (PDT) or as a fluorescence marker in diagnostics [1–6]. Because of its relatively high photostability and uncomplicated extraction from green plant leaves, pheophorbide a and its derivatives are used as model compounds for different photobiophysical investigations, such as the study of primary processes of photosynthesis or the molecular mechanism of photodynamic activity [7, 8].

The photophysical and photosensitizing properties of pheophorbide a (Pheo) are generally well known. This phorbide has been under investigation in our group since 1984. We have studied its electronic properties in organic solvents [9–11] and in microheterogeneous environments [12–14] and its photosensitizing activity *in vitro* and *in vivo* [4, 15]. The aim of this paper is to summarize today's knowledge about the electronic properties of Pheo in different environments.

The photophysical parameters of Pheo monomers were investigated in a number of papers [8, 10, 16, 17]. In the Jablonski diagram shown in Fig. 2 these parameters are summarized. In previous studies the singlet oxygen quantum yield of Pheo monomers was found to be $\Phi_\Delta = 0.59$ [16]. Since Pheo is a hydrophobic compound, it tends to aggregate in aqueous environments. The most important photophysical parameters for biological activity, such as fluorescence quantum yield (Φ_{Fl}) and singlet oxygen quantum yield (Φ_Δ), are strongly influenced by aggregation processes. The quantum yield of both processes decreases dramatically ($\Phi_{Fl} = 0.02$ and $\Phi_\Delta < 0.01$) in phosphate-buffered solution (PBS). On the other hand, *in vitro* as well as *in vivo* high photodynamic activity was observed. Considering these facts, it was not clear whether the dye deaggregates completely to monomers or whether non-photoactive equilibrium dimers also exist in cell membranes. If such dimers exist according to the findings of Dietel and Wendenburg [18], we have to take into account that during irradiation the equilibrium is shifted to monomers, giving an increasing amount of photosensitizing molecules. Cell suspen-

*Correspondence to: B. Röder, Humboldt-Universität zu Berlin, Institut für Physik, AG Photobiophysik, Invalidenstrasse 110, D-10115 Berlin, Germany.
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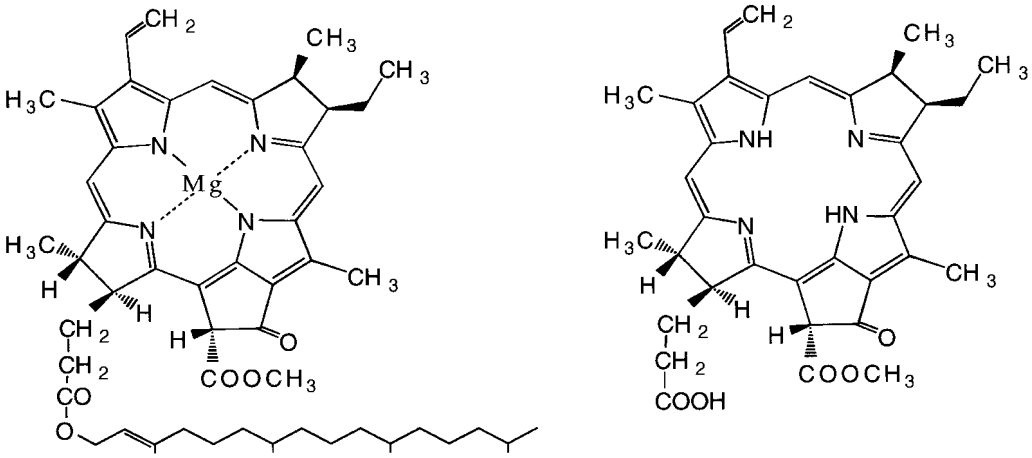

Fig. 1. Structural formulae of chlorophyll a (left) and pheophorbide a (right).

sions [19] incubated with Pheo show a strong fluorescence in the red region of the spectrum. Also *in vivo* strong Pheo fluorescence of tumours was observed [20]. The spectral distribution of the fluorescence was identical with the monomer spectrum (with some slight bathochromic shift due to the weak interaction with the surroundings).

MATERIAL AND METHODS

Photosensitizer

Pheophorbide a was prepared in our laboratory from leaves of *Urtica urens* according to Willstätter and Stoll [21]. In order to improve the yield and the separation from pheophorbide b respectively, we have introduced two modifications: first, the extraction was carried out using a Soxhlet extractor instead of a beaker; second, the acidic solution was not only diluted by water but also neutralized by aqueous sodium hydroxide.

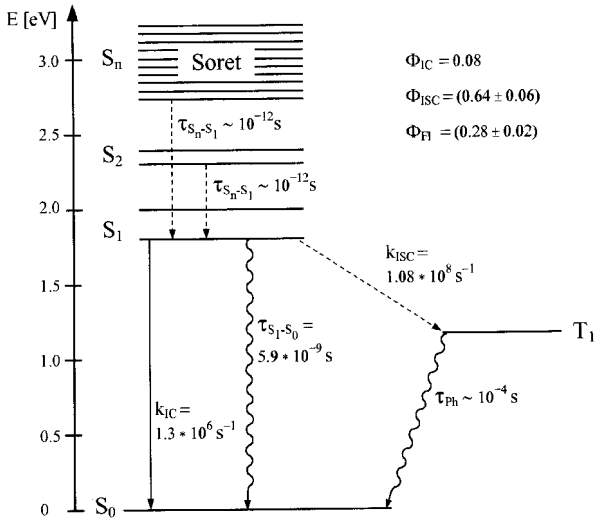

Fig. 2. Jablonski diagram of pheophorbide a (monomers).

Absorption Spectroscopy

The absorption spectra were measured using a Shimadzu 160A UV-vis spectrometer.

Fluorescence Spectroscopy

Steady state measurements were carried out using the self-made equipment described in Ref. 22. Time-resolved fluorescence and fluorescence anisotropy investigations were carried out on the TCSPC apparatus described in detail in Ref. 22. Steady state and dynamic anisotropies were calculated using the Perrin equation. Rotational diffusion constants were obtained according to $D = 1/6\tau_{rot}$.

Singlet Oxygen Detection

Steady state singlet luminescence and low-temperature steady state fluorescence anisotropy measurements were done using the self-made equipment described in Ref. 23. Time-resolved singlet oxygen luminescence was detected using the apparatus shown in Ref. 24.

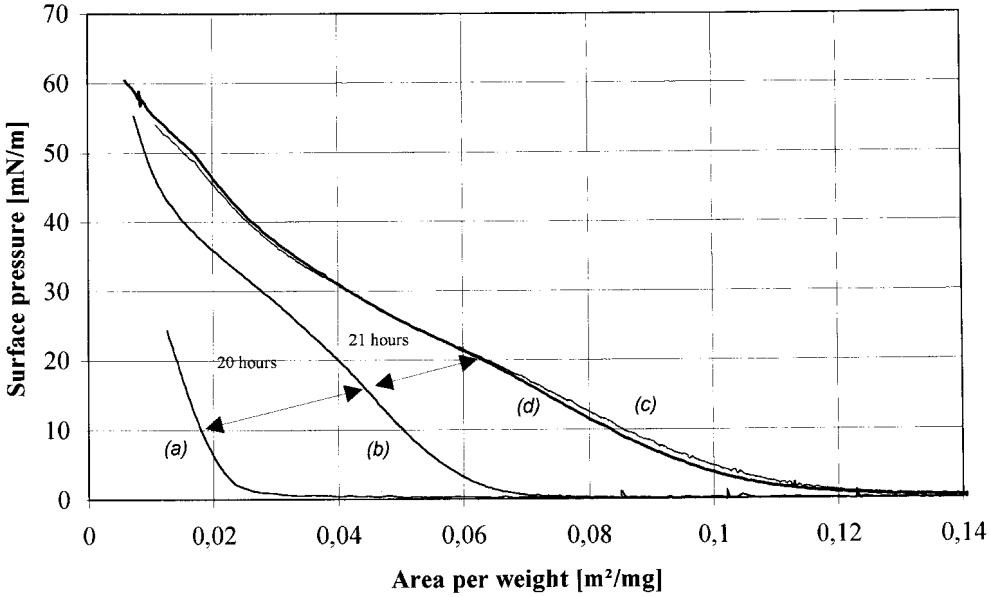

Fig. 3. Isotherms of OAT cell membrane fragments on a pure water subphase: (a) initial compression; (b) compression after 20 h; (c) compression after another 21 h; (d) compression after final 2 h. After each compression the layer was immediately expanded and allowed to rest. Owing to the fact that the molar weight is not available, the *x*-axis is based on the area per applied weight of the cell membrane fragments.

Erythrocyte Ghost Preparation

The preparation is given in detail in Ref. 24.

Langmuir–Blodgett (LB) Layers with Behenic Acid

Behenic acid (docosanic acid, $C_{22}H_{44}O_2$, Aldrich) was used without further purification, dissolved in chloroform (trichloromethane, spectroscopic grade, Aldrich).

The layers were formed and transferred using a commercially available film balance (FW2, Lauda, Germany) with a controlled trough temperature ($18 \pm 1\,°C$). The subphase consists of ultrapure water from a coupled reverse osmosis/milli-Q device (Millipore, France) with a salt concentration of about 5×10^{-4} mol l^{-1} $CdCl_2$ (Riedel deHaen). The samples were spread using a microsyringe (Hamilton). The pheophorbide a concentration in the spreading solution amounts to about 2.5×10^{-4} mol l^{-1} for mixed monolayers with behenic acid. Here the mole fraction x_{dye} is 0.1, meaning a ratio of one molecule of pheophorbide a to nine molecules of behenic acid. The components of mixed monolayers are spread together in one solution that is mixed immediately before the experiment.

Langmuir–Blodgett films are formed by transfer of the floating monolayer onto quartz plates ($10 \times 25 \times 1\,mm^3$, Medizinisch-Glastechnische Werkstätten, Berlin). The plates are cleaned by hot chrome sulfuric acid, hydrophilized by treatment with a mixture of concentrated sulfuric acid and hydrogen peroxide (3:1, v/v), then hydrophobized using dimethyldichlorosilane.

The monolayer transfer is performed by the vertical dipping technique using a film lift (FL1, Lauda, Germany). For further spectroscopic studies, only samples with a transfer coefficient of unity or about unity are used.

Because of the Cd^{2+} ion-containing subphase, the transferred layers are cadmium behenate layers rather than behenic acid layers. Nevertheless, in the present paper the name 'behenic acid layer' is used.

Langmuir–Schaefer (LS) Layers of Cell Membrane Fragments

The OAT cell membrane fragments were kindly provided by Professor Dr J. Moser from the Institute of Laser Medicine (Heinrich-Heine-Universitüt Düsseldorf). As spreading solution the usual stock solution was used. The solvent is a mixture of glycerol/HEPES (*N*-(2-hydroxyethyl)piperazine–*N*′-2-ethanesulfonic acid) buffer (50 mM) in a volume ratio of 1:1. The

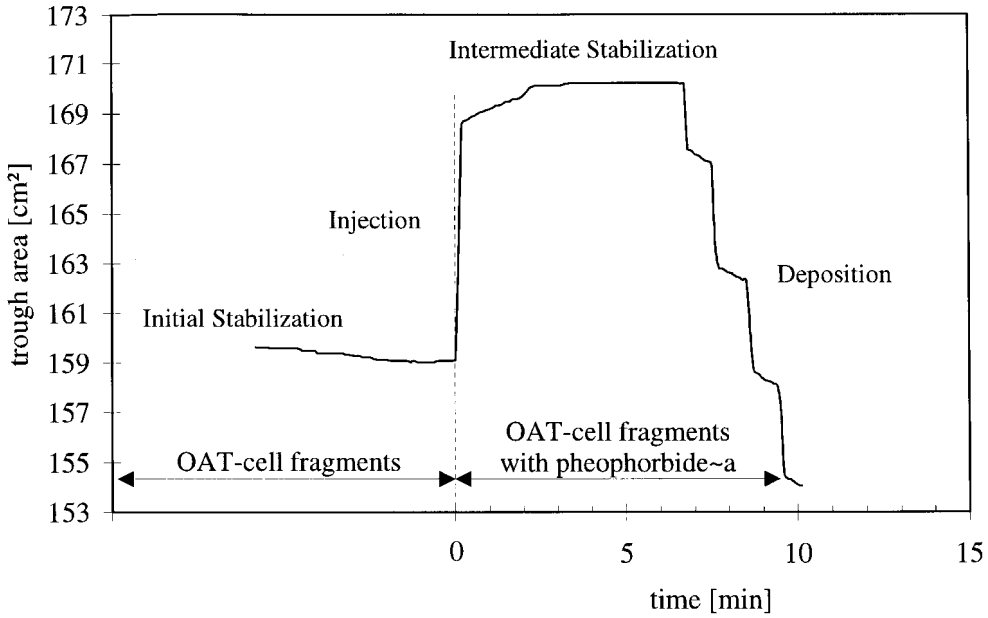

Fig. 4. Area versus time diagram of OAT cell membrane fragments, including last part of initial stabilization, injection of ethanolic pheophorbide ($t = 0$), stabilization of Pheo-containing layer and deposition. At the arbitrarily chosen point $t = 0$ the layer has been about 43.5 h on the subphase and has undergone four compression/expansion cycles and final compression to a constant surface pressure of 18 mN m^{-1} stabilization has taken 35 min.

concentration amounts to 0.9 mg ml^{-1}. Owing to the inhomogeneous, not molecularly dispersed and water-soluble spreading solution, the spreading as well as film-forming behaviour of the sample was complicated. Generally, film formation could not be guaranteed.

For recording isotherms, 0.45 ml of this solution was spreaded dropwise by a microsyringe. After recording an isotherm (Fig. 3(a)), the monolayer was expanded and the layer on the subphase was allowed to rest. The compression was repeated three times and then after 20 h, a further 21 h and a final 2 h (Fig. 3). The shape of the isotherms changes with time, but the last two isotherms are nearly identical. Obviously the membrane fragments are first suspended in the subphase, then undergo a sort of restructuring and finally form a stable layer. Here we used a constant surface pressure of 18 mN m^{-1}. After about 30 min this layer is stable and ready to incorporate pheophorbide molecules. For this purpose an ethanolic solution of Pheo is injected beneath the subphase. As shown in Fig. 4, the substance is incorporated immediately into the layer. After a short time this dye-containing layer is stabilized too. The deposition of the layer on a quartz substrate was carried out according to the Langmuir–Schaefer technique. Here the quartz plate is held horizontally, lowered till it touches the monolayer and then lifted. During each cycle a Pheo-containing double layer is transferred to one side of the quartz plate. The plates and their preparation are the same as used for Langmuir–Blodgett layers.

RESULTS AND DISCUSSION

Knowing the behaviour of Pheo in homogeneous solution, we investigated its photophysical properties in different microheterogeneous environments, namely in liposomes, micelles (Table 1), cyclodextrins and pheophorbide a–antibody complexes [14, 25]. In order to discuss the behaviour of Pheo in model membranes, the electronic properties of the dye embedded in liposomes or micelles are of interest. As shown in Table 1, in both systems a slight bathochromic shift of both the absorption and fluorescence spectra was observed. The fluorescence decay time becomes longer in liposomes owing to the hydrophobic environment of the dye. The decay time in micelles is similar to the decay observed for monomers in ethanol. In both systems singlet molecular oxygen is generated (Table 1).

Table 1. Selected photophysical parameters[a] of Pheo in different microheterogeneous environments

Environment	λ_{max} (abs.) ($Q_{(0,0)} - Q_{(1,0)}$) (nm)	λ_{max} (fluor.) (nm)		τ_{Fl_1} (ns)	τ_{Fl_2} (ns)	D (s^{-1})	Φ_Δ
Ethanol[b] [14]	667	675	720	5.9 ± 0.1		7.6×10^8	0.59
PBS[c] [14]	683	667	710	3.7 ± 0.1	0.2 ± 0.1		0.02 max.
Liposomes [14]							I_Δ/I_Δ (eth.)[d]
Pheo[b]:lipid							
1:50	669	680	725	6.2 ± 0.1			No SOL
1:200	669	680	725	6.2 ± 0.1		1.2×10^8	0.23
1:500	669	680	725	6.2 ± 0.1			0.33
1:1100	669	680	725	6.2 ± 0.1			0.51
1:1700	669	680	725	6.2 ± 0.1			0.70
PBS +1% Triton X-100 [14]	669	691	725	5.9 ± 0.1		3.5×10^7	SOL
LB films	707	675	720	3.7 ± 0.4	0.15 ± 0.03		No SOL
LS films	677/702	679	735	3.9 ± 0.4			No SOL
EGS	675 [24]	680	725	6.1 ± 0.1			SOL

[a] τ_{Fl}, fluorescence decay time; D, rotational diffusion constant; Φ_Δ, singlet oxygen quantum yield.
[b] $c_{Pheo} = 1 \times 10^{-5}$ M.
[c] pH 7.4; $c_{Pheo} = 1.5 \times 10^{-5}$ M; $T = 348$ K; 5% ethanol.
[d] No quantum yield was determined. The ratio between the luminescence intensity of the system liposome/Pheo and of Pheo in ethanol is shown.
[e] SOL, singlet oxygen luminescence detected.
[f] No SOL, no singlet oxygen luminescence detected.

Based on steady state and time-resolved fluorescence anisotropy measurements, some information about the microenvironment of dye molecules can be obtained. In a first step the steady state anisotropy of Pheo was measured to obtain the average anisotropy and the angle between the dipole orientation in the ground and the various excited states of Pheo. The results are summarized in Fig. 5 and Table 2.

For the most interesting transition $S_{0,0} \rightarrow S_{1,0}$ an average anisotropy of $r = 0.26$ was found. The results summarized in Table 2 are in good agreement with parameters obtained for pheophytin a [26]. In further time-resolved experiments the rotational diffusion time (D) of Pheo in different microheterogeneous environments was determined (Table 1). We found that Pheo as hydrophobic dye is located inside the hydrophobic part of liposomes and included in micelles probably in its monomeric form [14]. However, this information is not sufficient for some prediction of the behaviour of Pheo in biological membranes. These membranes consist not only of lipids but also of cholesterol, proteins, glycoproteins, glycolipids and other compounds. For this reason we focused our interest on the investigation of membrane models closer to biological reality: erythrocyte ghost suspensions (EGS) and Langmuir layers containing membrane fragments of cancer cells.

In this paper we will focus on the following aspects of the above problem.

1. Is it possible to extract the dimer spectrum of pheophorbide a in solution and in membrane-like structures to determine the electronic parameters of the dimer?
2. How do microheterogeneous environments modelling different aspects of the cell environment influence the electronic properties of pheophorbide a?
3. Is it possible to develop a membrane model which yields similar properties to biological membranes and could be investigated by physical methods?

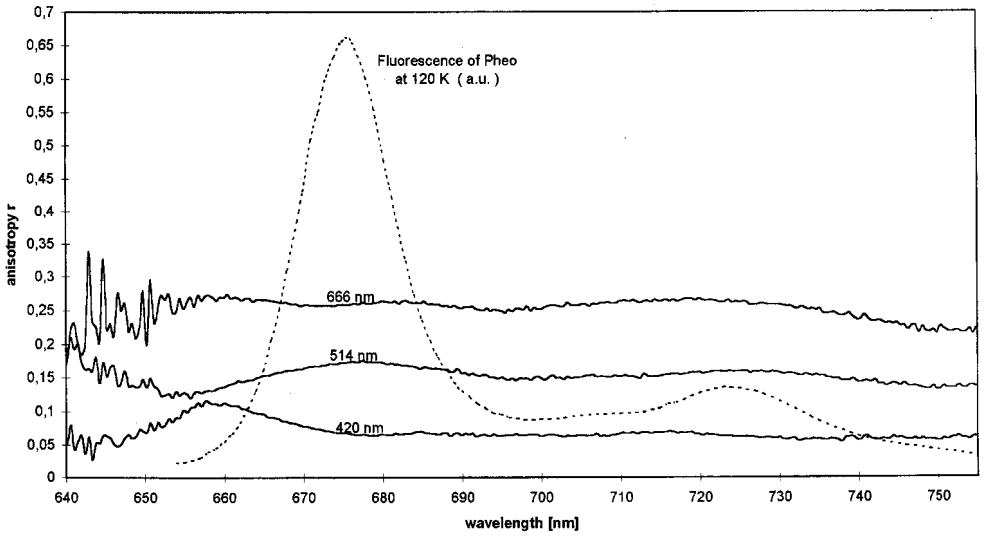

Fig. 5. Fluorescence spectrum of Pheo at 120 K and steady state fluorescence anisotropy at different wavelengths.

Table 2. Average anisotropy and angle (α) between absorption and fluorescence dipole moment for different electronic transitions of Pheo

λ_{exc} (nm)	Electronic transition	Average anisotropy	α (deg)
666	$S_{0,0} \rightarrow S_{1,0}$	0.26	29
514	$S_{0,0} \rightarrow S_{2,0}$	0.16	39
420	$S_{0,0} \rightarrow S_n$	0.06	49

Properties of Pheophorbide a Equilibrium Dimers in Solution

The preparation of pheophorbide monomer–dimer equilibria in solution was realized at dye concentrations of about 10^{-5} M. At these concentrations the solution does not contain higher aggregates, as was shown previously by temperature-dependent investigation of the fluorescence quantum yield [27].

By varying the temperature and the ethanol/water ratio in the solution at constant dye concentration, we observed a set of absorption spectra containing isosbestic points [28]. Analysing these spectra, we obtained linear extinction difference diagrams suggesting a first-order reaction. The analysis of these results, the knowledge of the pure monomer spectrum from measurements in ethanol, resulted in the extraction of the dimer spectrum with an absorption maximum at 685 nm [28]. Using exciton theory for simulation of the dipole–dipole interaction between two identical molecules, we calculated an absorption maximum of the dimer at $\lambda_D = 684$ nm with a dipole moment of $M = 3$ Debye [28]. These very closely corresponding results also suggest that no higher aggregates are present in the investigated samples.

Fluorescence investigations of Pheo in mixtures of ethanol and PBS show that with increasing content of PBS two fluorescence decay times can be observed [10, 14]. It can be seen that the decay times and amplitudes of the two fluorescence components depend strongly on the content of PBS, i.e. of monomers and aggregates. In PBS with 5% ethanol the fluorescence intensity is very low and the fluorescence lifetime of the monomers is shortened to $\tau_1 = 3.7$ ns, probably because of energy transfer processes between monomers and aggregates. A second component with a lifetime of $\tau_2 = 170$ ps is also observed [10, 14, 28].

Interaction of Pheo Molecules in Langmuir–Blodgett Films

In a first step, Langmuir–Blodgett layers of pure Pheo and of a mixture of Pheo and behenic acid were prepared. The central part of the coated area exhibited very constant spectral properties, as was shown previously [22]. In all experiments the observation area was located in this part of the film. Upon investigating a number of samples, we found that the

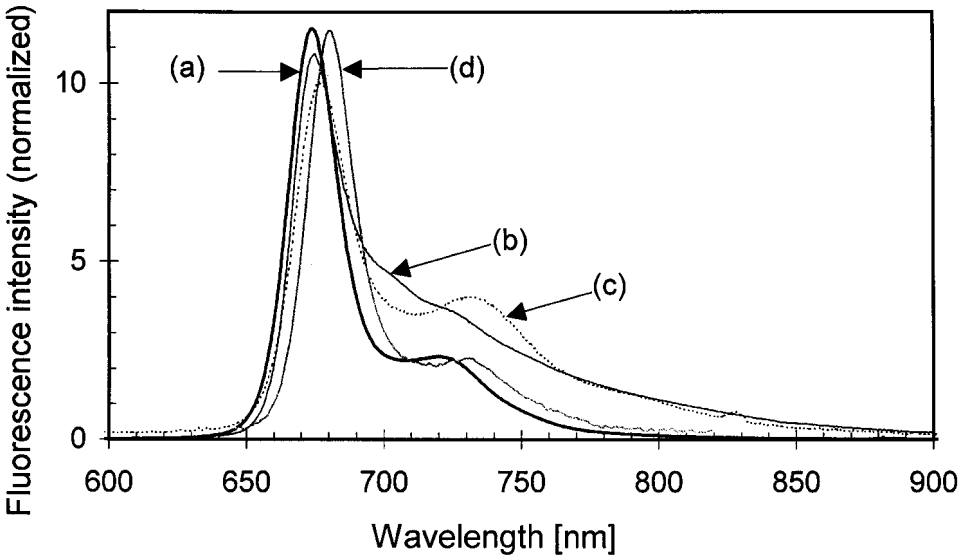

Fig. 6. Fluorescence spectra of Pheo in different environments: (a) in ethanol; (b) in Langmuir–Blodgett monolayer with behenic acid (1:9) on a quartz plate; (c) in Langmuir–Schaefer multilayers with cell membrane fragments on a quartz plate; (d) in erythrocyte ghost suspension (EGS).

pure Pheo layer shows the spectral behaviour of an aggregated dye whilst the mixed layer exhibits a monomer fluorescence spectrum. Detailed investigation and photophysical characterization of mono- and multilayers of pheophorbide a embedded in behenic acid showed that excitonic interaction occurs [29] between Pheo molecules in two neighbouring layers in which the layers are oriented with the polar head groups of the behenic acid molecules one to the other. The application of exciton theory to this problem resulted in a maximum interaction at a stacking angle of 39° between the two molecules and the same spectral shift of absorbance as for equilibrium dimers in solution [28]. Also two fluorescence decay times were observed: $\tau_1 = 3.7$ ns and $\tau_2 = 150$ ps. These findings are in full agreement with the results obtained for monomer–dimer equilibria in ethanol/water mixtures. No singlet oxygen luminescence could be detected in the layers.

Langmuir–Schaefer Multilayers

In a second step, Langmuir–Schaefer multilayers with cell membrane fragments were prepared. In Fig. 6 the fluorescence spectra of Pheo in different environments are shown. The shape of the spectrum of Pheo in Langmuir–Schaefer layers is much closer to that of the Pheo monomer in ethanol than to that of Pheo in Langmuir–Blodgett layers.

The fluorescence decays monoexponentially with $\tau_1 = 4$ ns. These observations suggest that Pheo exists in the OAT membrane layers mainly in its monomer form and that the dye molecules are in strong interaction with the surroundings, leading to the short fluorescence lifetime. Because of this, it can be proposed that no efficient interaction between Pheo molecules occurs in this system. No singlet oxygen luminescence could be observed.

Photophysical Parameters of Pheophorbide a in EGS

The incorporation of Pheo in ghost cell membranes was studied by absorption measurements in Ref. [24]. The fluorescence spectrum is similar to that obtained in liposomes (not shown, Table 1) and to that in ethanol (Fig. 6, Table 1), with a slight bathochromic shift due to the interaction with the surroundings. In contrast with the time-resolved fluorescence measurements in Langmuir layers, for Pheo in ghost cell membranes a fluorescence decay time of $\tau_{F1} = 6.0 \pm 0.1$ ns was observed. This decay time is between the decay times obtained for Pheo in ethanol and micelles ($\tau_{F1} = 5.9 \pm 0.1$ ns) on the one hand and in liposomes ($\tau_{F1} = 6.2 \pm 0.1$ ns [14]) on the other hand. In this case, also singlet oxygen luminescence is observed [24].

CONCLUSIONS

The experimental data obtained for Pheo in homogeneous solution and in different microheterogeneous environments allow us to deduce that Pheo is incorporated in cell membranes mainly in its monomer form. The results also show that simple Langmuir layers as employed in our experiments cannot be used as a model for the study of photosensitizer behaviour in cell membranes. In contrast, erythrocyte ghosts are promising model systems for the study of primary photoprocesses of hydrophobic photosensitizers.

It seems that the content of proteins and other molecules different from lipids as well as lipid composition and the aqueous surroundings play an important role in the electronic properties of Pheo incorporated in cell membranes. Further investigations on this topic using erythrocyte ghost suspensions as models for biological membranes are in progress.

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