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Solvent relaxation behaviour of *n*-anthroyloxy fatty acids in PC-vesicles and paraffin oil: a time-resolved emission spectra study

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Abstract

Time-resolved fluorescence measurements were performed for a set of *n*-anthroyloxy fatty acids (*n*-AS; $n = 2, 3, 6, 9, 12, 16$) in both solvent and vesicle systems. The Stokes' shifts and the mean relaxation times calculated from the time-resolved emission spectra (TRES) are shown to be strongly dependent on the position of the fluorophore in small unilamellar vesicles (SUV) composed of phosphatidylcholine (PC), while they are essentially independent of the fluorophore position in isotropic paraffin oil. The concept of an intramolecular relaxation process which had been suggested to explain the wavelength dependence of the emission behaviour of the *n*-AS dyes in viscous solvents is supported by semiempirical calculations showing that a more planar conformation is favoured in the excited compared to the ground state. However, in order to explain the results in vesicle systems, the concept of intramolecular relaxation is not sufficient. Rather, we show that intermolecular solvent relaxation processes play the dominant role for the wavelength dependent emission behaviour in polar, viscous environments.

Keywords: *n*-Anthroyloxy fatty acid; Solvent relaxation; Intramolecular relaxation; Semiempirical calculation; Small unilamellar vesicle; Time-resolved emission spectroscopy

1. Introduction

The validity of the information gained by the probe approach in studies of natural and artificial membranes depends on three factors [1]: (i) the location of the probe must be known, (ii) there must be no major perturbations by the probe and (iii) the probe must be sensitive to changes in the membrane properties. The set of *n*-anthroyloxy fatty acids (*n*-AS) constitute an unique set of fluorescent dyes with a common chromophore covalently attached at different positions ($n = 2, 3, 6, 9, 12, 16$) along the acyl chain of the fatty acid (stearic acid for $n = 2–12$; palmitic acid for $n = 16$). The *n*-AS probes are known to insert into the membrane with the stearoyl chains parallel to the phospholipid acyl chains with the long axes of the anthroyl ring on average almost perpendicular to the plane of the membrane [2]. The chromophore shows little motion about the ester linkage and is positioned at a defined depth of the bilayer as confirmed by both ^{1}H-NMR [3] and quenching studies using Ca^{2+} and dimethylamine [4]. Therefore, the

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series of *n*-AS has been used extensively in membrane biophysical studies, for example to monitor fluidity changes in lipid bilayers as a result of bulk transitions such as a gel to liquid crystalline phase transition [4–8], to prove the existence of a polarity gradient along the bilayer normal [9] or as acceptors in determining the location of Trp-residues in membrane proteins [10–12].

The photophysics of 9-anthroic esters have been first studied by Werner et al. [13–16]. It was concluded that the ground state should be non-planar due to steric hindrance between the carboxyl group and the ring peri hydrogens. The considerable increase in the dipole moment after excitation [14] yield an electronic state with strong charge transfer character (S_1^{FC}) which is thought to relax to an emissive state with a more planar geometry (S_1^{EQ}). No shifts were observed in excitation spectra for 9-AS regardless of the solvent used, showing that the ground state interaction of the fluorophore with its surrounding is small. Thus changes seen in the emission spectra were attributed to excited state processes [17].

The decay behaviour in homogeneous fluid solvents is monoexponential and constant across the emission spectrum. In viscous media, like paraffin oil or in phospholipid bilayers, however, the fluorescence decay becomes non-exponential and strongly dependent on the emission wavelength. The increase in mean decay times [18] with increasing emission wavelength was first described by Matayoshi and Kleinfeld [5] and taken as evidence for a correlation between an excited state rotation rate and solvent viscosity. A two-state model has been proposed, with the two states corresponding to two different conformers. The relaxation of the initially excited Franck–Condon state (S_1^{FC}) to the state with equilibrium geometry (S_1^{EQ}) is assumed to involve the rotation of the carbonyl group from a perpendicular orientation (relative to the plane of the anthracene ring) towards a more coplanar position. Studies with 12-AS in paraffin and mineral oil [5,19] show that an excited state spectral shift occurs in absence of dielectric relaxation, suggesting that an intrinsic relaxation process exclusively accounts for the wavelength dependence of the fluorescence decay behaviour.

It seems to be questionable, however, whether the simple model of intramolecular relaxation can be sufficient to explain the decay and relaxation behaviour of the series of *n*-AS in phospholipid bilayers, as was assumed by Matayoshi and Kleinfeld. Phospholipid membranes constitute a highly anisotropic medium with water molecules penetrating into the hydrophobic alkyl chain region [9,20,21]; thus, the relaxation behaviour of the *n*-AS dyes should depend on their position within the membrane. Together with the charge transfer character of the anthroyl chromophore, the presence of the highly polar solvent water may lead to the suspicion that dielectric solvent relaxation contributes to the fluorescence relaxation behaviour of *n*-AS incorporated in membranes. The important role of bilayer water and its possible participitation in membrane relaxation processes has also been stressed by several theoretical studies [22,23].

Thus, we decided to reinvestigate the relaxation dynamics of the series of *n*-AS in greater detail by the use of time-resolved emission spectra which allow to monitor directly the excited state behaviour as function of time. The calculation of correlation functions from the time-dependent emission maxima yield a quantitative description of the relaxation process. In the present work the relaxation behaviour of 2-, 6-, 9-, 12-AS and 16-AP is studied in both different solvent systems (paraffin oil, 3-methylpentane, ethanol) and small unilamellar vesicles prepared of phosphatidylcholine (PC), using the reconstruction method of Maroncelli and Fleming [24]. We show that the relaxation behaviour is quite unsensitive to the position of the fluorophore in paraffin oil and 3-methylpentane, while it depends strongly on the position in PC-vesicles, indicating that an intramolecular relaxation process is not sufficient to explain the observed behaviour in vesicles. In contradiction to the state of understanding up to now we provide evidence that dipolar solvent relaxation is the excited state process predominantly determining the fluorescence behaviour of the set of *n*-AS dyes in vesicle systems.

2. Materials and methods

Spectroscopic grade solvents (paraffin oil, 3-methylpentane, ethanol) were obtained from Merck; egg-yolk phosphatidylcholine (PC) and bovine brain phosphatidylserine (PS) were supplied by Fluka. The

n-anthroyloxy fatty acids were purchased from Molecular Probes. All other chemicals were of the highest purity commercially obtainable. Small unilamellar vesicles (SUV) were prepared essentially as described by Bashford et al. [1]. In brief, the respective *n*-AS dye (in EtOH) was added to the lipid in $CHCl_3$ to yield a lipid/dye ratio of 100:1. The solvent was removed under a stream of N_2 and the lipid-dye mixture was further dried under vacuum overnight. After addition of Tris-buffer (pH 7.5, 100 mM NaCl) the lipid suspension (1 mM) was sonicated at room temperature for 10 min, allowed to anneal for 30 min [25] and centrifuged for 15 min to remove titanium particles. Steady-state spectra were recorded using an Aminco Bowman II spectrometer. Fluorescence decays were recorded with commercial single photon counting equipment (Edinburgh Instruments 199S) and analysed using an iterative reconvolution technique, as described elsewhere [26]. Mean decay times were calculated according to

$$\langle \tau \rangle = (A_1\tau_1 + A_2\tau_2 + A_3\tau_3)/100 \quad (1)$$

where the τ_i are the individual decay times and the A_i are the corresponding relative amplitudes. Time-resolved emission spectra (TRES) were calculated from the fit parameters of the multiexponential decays detected from 390 to 530 nm and the corresponding steady-state intensities. The TRES were fitted by log-normal functions [27]. Correlation functions *C(t)* are calculated from the emission maxima $\nu(t)$ of the TRES at defined time t after excitation:

$$C(t) = \frac{\nu(t) - \nu(\ )}{\nu(0) - \nu(\ )} \quad (2)$$

where $\nu(0)$ and $\nu(\ )$ are the emission maxima (in cm^{-1}) at times zero and , respectively. In all cases the solvent response cannot be satisfactorily described by a monoexponential relaxation model. In order to characterize the overall time scale of the solvation response, we use an (integral) average relaxation time:

$$\langle \tau_r \rangle \equiv \int_0 C(t)\,dt \quad (3)$$

All semiempirical calculations were performed using the VAMP program version 5.6 [28] on a Silicon Graphics Power Challenge. The standard AM1 parameters for C, H and O were used [29]. The optimization of ground state geometries was done using Baker's eigenvector following algorithm [30]. Geometries in the first excited singlet state were calculated using a configuration interaction expansion of microstates. In order to reduce computational time, only single and pair excitations within the active space of 24 orbitals were used.

3. Results

The fluorescence behaviour of the series of *n*-AS was determined in the isotropic solvents EtOH (polar, fluid), 3-methylpentane (unpolar, fluid), paraffin oil (unpolar, viscous) and in PC-SUV (unisotropic, viscous) at 25°C across the emission spectrum from 390 to 530 nm in 10 nm steps.

3.1. Decay behaviour in EtOH

As expected, no dependence on either excitation wavelength (316, 337, 358, 380 nm) or emission wavelength (390 to 530 nm) was noticed in EtOH; a monoexponential decay behaviour was observed in all cases. However, the decay times were found to be dependent on the position of the fluorophore along the stearyl chain: while the decay time was essentially identical for 6-, 9- and 12-AS ($\tau \approx 3.0$ ns), the decay is considerably slower for 3-AS ($\tau = 5.8$ ns) and even more for 2-AS ($\tau = 7.6$ ns).

3.2. Decay behaviour in 3-methylpentane

The decay behaviour is largely unaffected by the position of the fluorophore in 3-methylpentane, but becomes dependent on the emission wavelength. A biexponential decay law was necessary to describe the decay behaviour at the blue edge of the emission spectrum, the mean decay time increasing about 1.5 ns from 390 to 420 nm. At longer wavelengths the decay is monoexponential and the decay times do not further increase with λ_{em}.

3.3. Decay behaviour in paraffin oil

A more complex behaviour is observed in the viscous paraffin oil. While a biexponential model was

satisfactory near the emission maximum and at longer wavelengths, three exponentials were necessary to fit the data at the blue edge of the spectrum, in agreement with [19]. A negative pre-exponential factor is always observed for $\lambda_{em} \geq 470$ nm, suggesting the occurrence of an excited state reaction [18]. For 6-, 9- and 12-AS the decay behaviour in paraffin oil was very similar. The mean decay times increase continuously with increasing wavelengths, with the most pronounced increase between 390 and 420 nm. The decay times of 2-AS are about 1 ns to 1.5 ns longer than those of the other AS-dyes, presumably due to some intramolecular interaction of the carboxyl group with the ester group [31].

3.4. Decay behaviour in PC-SUV

A biexponential model has been commonly used to describe the decay behaviour of the *n*-AS in model membrane systems [5,9,21]. This yields a good fit if the emission is detected in the region of the emission maximum, as it has been done in most studies. However, we found that a biexponential fit was not satisfactory at the blue edge of the spectrum (390–410 nm); here, a three-exponential model gave a significant better fit, as may be judged from the residual plots in Fig. 1. For emission wavelengths ≥ 470 nm one component with a negative pre-exponential factor was always obtained. A strong increase of the mean decay times with emission wavelength was observed which is more than 100% for 2-AS (Fig. 2). In addition, the mean decay time in PC-SUV depend on the position of the anthroyloxy chromophore in a systematic way, increasing with the depth of incorporation. This result, observable at any detected wavelength, is in accordance with previous work on the group of *n*-AS in vesicle systems which has been restricted to detection at a single wavelength in most cases [4,32,33].

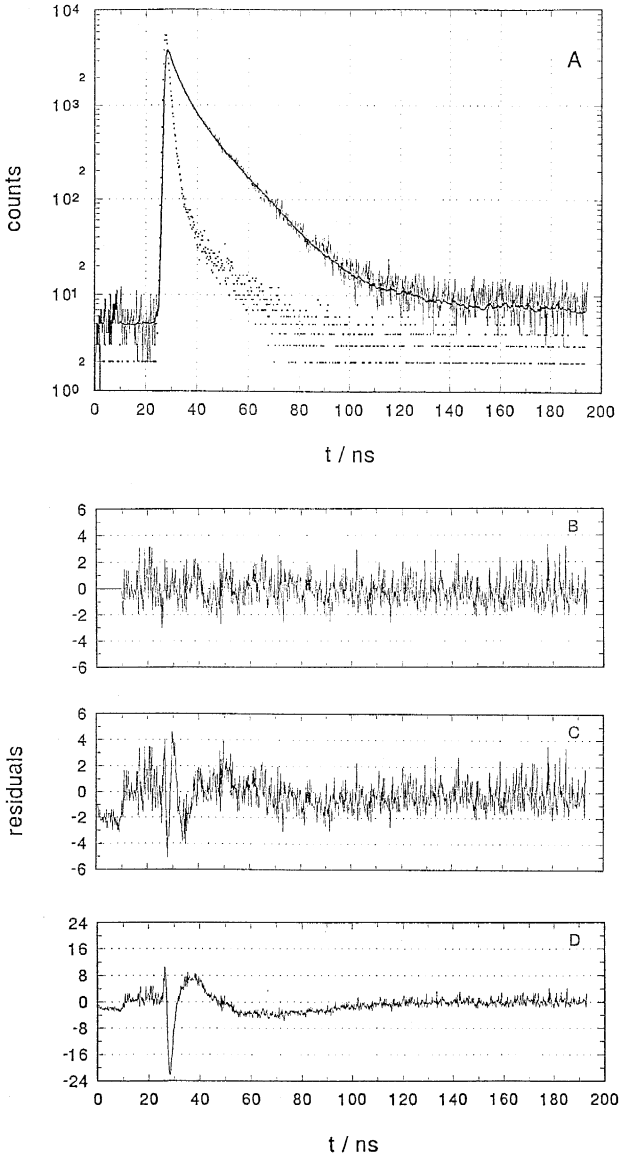

Fig. 1. Fluorescence decay and residuals for 12-AS in PC-SUV. The excitation wavelength was 358 nm; emission was detected at 390 nm. (A) Lamp profile (dots), fluorescence decay and three-exponential fit (bold line). (B) Residual plot for three-exponential decay model. (C) Residual plot for biexponential decay model. (D) Residual plot for monoexponential decay model.

3.5. Time-resolved emission spectra (TRES) in isotropic solvents

The decay parameters obtained from the multiexponential fits together with the steady-state intensities at the corresponding wavelengths have been used to calculate the TRES. As expected, no spectral shift could be observed for 2-AS in EtOH. With the given time resolution the totally relaxed spectrum is observed independent of the time after excitation.

In 3-methylpentane a small shift (≈ 3 nm) was observed for 2-, 6-, 12-AS and 16-AP (not shown) between 0.2 and 20 ns which was considered to small for further interpretation. Considerably larger shifts with time after excitation of ≈ 7–10 nm were ob-

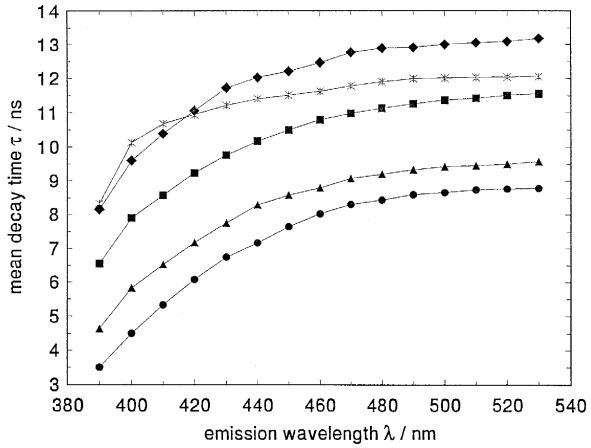

Fig. 2. Mean decay times, calculated according to Eq. (1) for the set of *n*-AS dyes in PC-SUV as a function of emission wavelength. The excitation wavelength was 358 nm; temperature was 25°C. Circles: 2-AS; triangles: 6-AS; boxes: 9-AS; diamonds: 12-AS; asterisks: 16-AP.

served in paraffin oil, essentially independent of the position of the chromophore. The spectra of 2-AS are red-shifted compared with the other *n*-AS and show a somewhat smaller shift. This behaviour is summarized in Fig. 3, showing the emission maxima (in cm^{-1}) as function of time after excitation. Since the absolute magnitude of the shift is small the construction of correlation functions seems to be a non-valid approach for the characterization of the relaxation dynamics. However, from Fig. 3 it can be concluded that the overall solvent relaxation behaviour in paraffin oil is very similar for all *n*-AS.

3.6. Time-resolved emission spectra in PC-SUV

A completely different relaxation behaviour was obtained in PC-vesicles. While the Stokes' shift is small (≈ 5 nm) and comparable to that observed in paraffin oil for 16-AP a large increase of the shift is observed in the series 16-AP < 12-AS < 9-AS < 6-AS < 2-AS (Fig. 4). With 2-AS a Stokes' shift of ≈ 40 nm was obtained within the first 20 ns after excitation. After this time, the relaxation is essentially complete for all dyes, as may be seen in Fig. 5, summarizing the increases of the emission maxima as a function of time after excitation. In Fig. 6 the corresponding correlation functions are presented. Both the absolute value of the Stokes' shift and the rate of the relaxation process decrease when increasing the depth of incorporation of the fluorophore (Table 1). As the Stokes' shifts in paraffin oil were much smaller and independent of the fluorophore position, we may assume that a second relaxation process, which is not observable in paraffin oil, contributes to the relaxation behaviour in PC-SUV.

3.7. Time-resolved emission spectra in PC-SUV containing phosphatidylserine (PS)

In a membrane binding study of prothrombin fragment 1 [34] we have shown that the relaxation behaviour of the two polarity sensitive probes Prodan and Patman is sensitive to the molar content of PS in mixed PC/PS-SUV, to the presence or absence of calcium ions and to the binding of prothrombin fragment 1, the N-terminal fragment of the vitamin K dependent protein prothrombin. On the basis of the described results with the series of *n*-AS it was interesting therefore, whether changes in the PC/PS molar ratio or the addition of Ca^{2+} would also affect the relaxation behaviour of the *n*-AS dyes at different depth in the bilayer. 6-AS and 12-AS were selected for a first study. TRES and the corresponding correlation functions were calculated for both dyes in vesicles containing 0, 20 or 50% PS in PC in absence or presence of 3 mM Ca^{2+}. The results are summarized

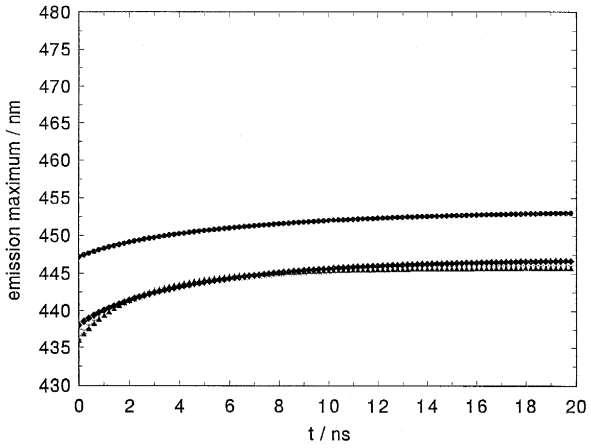

Fig. 3. Time-course of the emission maxima as a function of time after excitation of the *n*-AS in paraffin oil. Circles (upper line): 2-AS; triangles (lowest line): 6-AS; diamonds (top lower line): 12-AS; asterisks (middle line): 16-AP.

in Table 2. An increase in the mean relaxation times was observed with increasing PS-content, which is more pronounced for 6-AS being localized closer to the headgroup region. In presence of Ca^{2+}, a further increase in relaxation times is observed if at least 20% PS are present.

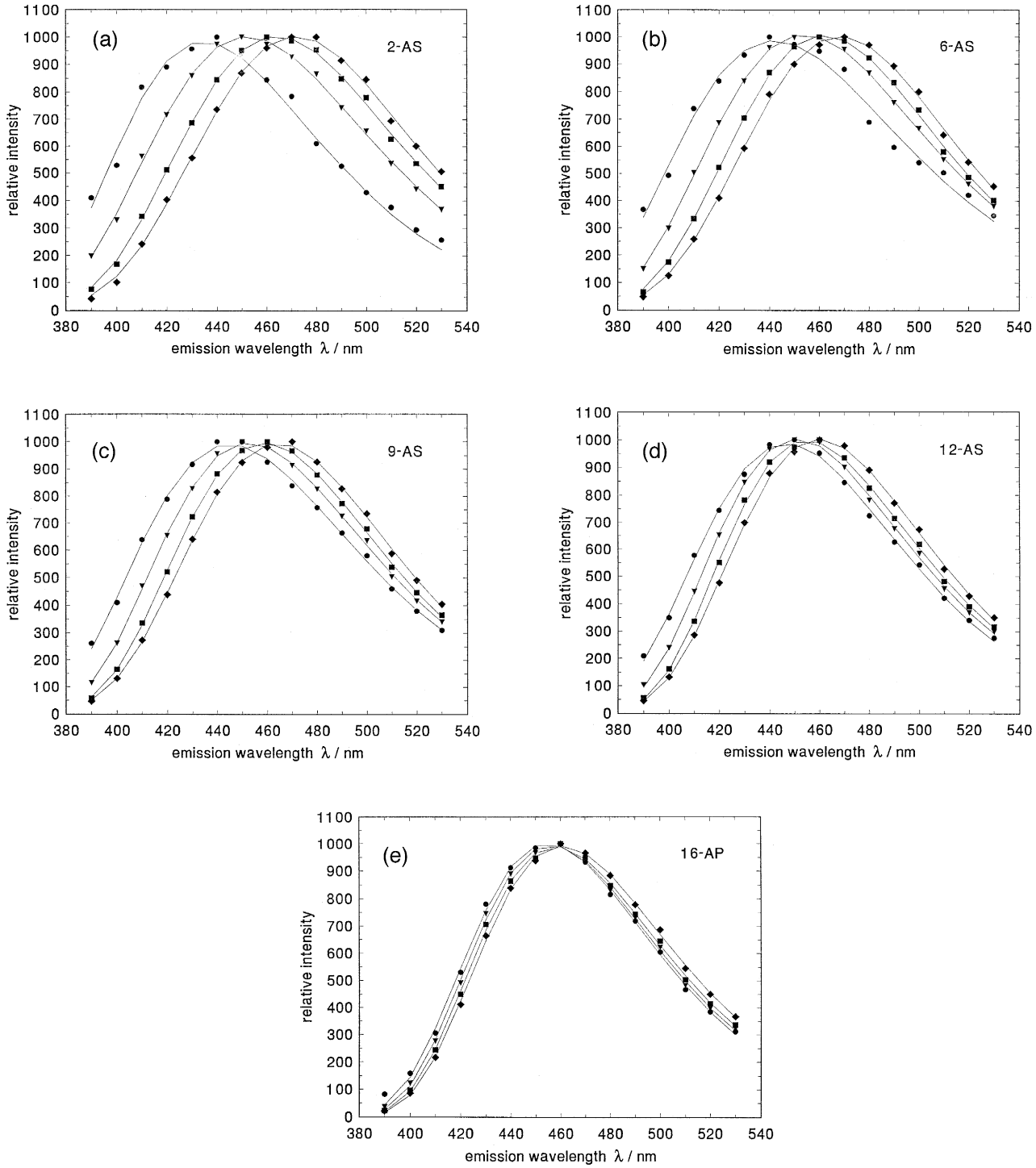

Fig. 4. Time-resolved emission spectra for the *n*-AS in PC-SUV. The respective chromophores are indicated inside the plots. Spectra are shown at 0.2 ns after excitation (circles), 2.0 ns (triangles); 5.0 ns (boxes) and 20.0 ns (diamonds).

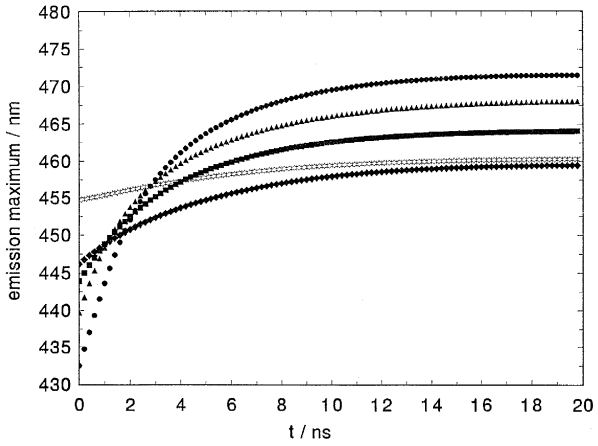

Fig. 5. Time-course of the emission maxima as a function of time after excitation of the *n*-AS in PC-SUV. Circles: 2-AS; triangles: 6-AS; boxes: 9-AS; diamonds: 12-AS; asterisks: 16-AP.

3.8. Semiempirical calculations

3.8.1. Formation of hydrogen bonds

Garrison et al. [17] have attributed the biexponential fluorescence decay of 2-AS in EtOH which they reported in their work to two different conformers of 2-AS with and without an intramolecular hydrogen bond. Semiempirical calculations are a valuable tool describing the relative stabilities of different conformations which a flexible molecule can occupy. If it is possible to form a hydrogen bond during the molecular motion, an additional stabilization might be expected. It has been shown that the AM1 method reproduces hydrogen bond energies, geometries and dipole moments of several hydrogen-bonded systems satisfactory [35].

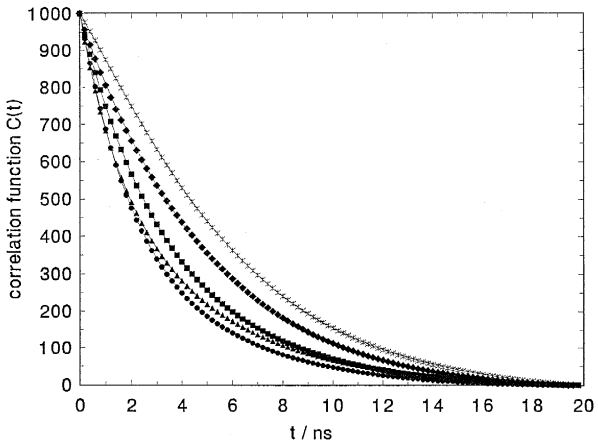

Fig. 6. Correlation functions *c(t)* calculated according to Eq. (2) from the emission maxima $\nu(t)$ for the *n*-AS dyes in PC-SUV. Circles: 2-AS; triangles: 6-AS; boxes: 9-AS; diamonds: 12-AS; asterisks: 16-AP.

Table 1
Stokes' shifts for $t = 20$ ns after excitation and mean relaxation times $\langle\tau_r\rangle$, calculated according to Eq. (3) for the *n*-AS dyes in PC-SUV

n	Shift (nm)	$\langle\tau_r\rangle$ (ns)
2	39	2.7
6	27	3.0
9	20	3.6
12	13	4.8
16	6	5.9

n represents the position of the chromophore along the acyl chain.

To investigate the effect of possible intramolecular hydrogen bonding on the relative stability of different *n*-anthroyloxy fatty acid conformers, we performed an analysis of the conformational space of the flexible side chain of the 2-AS model compound 2-(9-anthroyloxy)propionic acid (Fig. 7). Therefore, rotational energy profiles of the carboxyl function attached to the aromatic body with and without hydrogen bond to the carboxylic group of the propionic acid were calculated. The dihedral angle 4-3-2-1 was changed in 10° steps and held fixed; all other internal coordinates were fully optimized.

Table 3 shows the heats of formation (H_f) with corresponding dihedral angles (4-3-2-1) for different stationary points on the potential energy hypersurface in the electronic ground state (S_0) and in the first

Table 2
Percentual increase in the mean relaxation times for 6- and 12-AS with increasing PS content in presence and absence of calcium ions

System	Increase in $\langle\tau_r\rangle$ [a]	
	$-Ca^{2+}$	$+3$ mM Ca^{2+}
20% PS, 6-AS	17%	20%
50% PS, 6-AS	40%	49%
20% PS, 12-AS	6%	10%
50% PS, 12-AS	18%	19%

[a] $\langle\tau_r\rangle$represents the mean relaxation time, calculated according to Eq. (3). The percentual increases are relative to the systems with 100% PC.

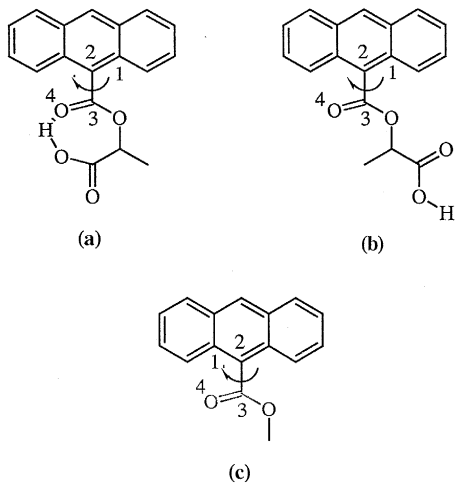

Fig. 7. Model compound for 2-AS (2-(9-anthroyloxy)propionic acid) with (a) and without (b) intramolecular hydrogen bond. 9-Methylanthroate (c) serves as a model for scanning the conformational behaviour upon electronic excitation. The arrows symbolize the reaction coordinates for scanning the conformational space.

excited singlet state (S_1). The transition state connects the two relative minima on the potential energy surface.

The presence of a hydrogen bond (Fig. 7a) allows the formation of two discrete local minima in the electronic ground and excited state, respectively (Table 3). The energetic separation of the corresponding minima is very small in the electronic ground state with a slight increase of the activation barrier between the minima in the excited state. If there is no hydrogen bond present (Fig. 7b), only one local minimum in each electronic state can be found. Both minima (S_0 and S_1) are energetically stabilized by ≈ 4 kcal/mol compared to the minima including a hydrogen bond. We therefore argument that even in the gas phase, where possible hydrogen bond interactions are not damped by solvent molecules, no tendency towards the formation of intramolecular hydrogen bonds can be found.

The transition state for the conversion from the non-hydrogen bonded to a state with a hydrogen bond in the electronic ground state was found at -97.67 kcal/mol. In the first excited singlet state, the exact location of the corresponding transition state was not possible yet. Nevertheless, there is strong evidence that the energy is not higher than -23 kcal/mol. A ground state activation energy of about 9 kcal/mol is not high enough to prevent rapid equilibration of both states at room temperature. These data clearly indicate that the observation of a biexponential decay behaviour cannot be interpreted in terms of two distinct populations of molecular conformations with and without hydrogen bond. Thus, there should be no influence on the fluorescence decay type resulting out of different excited state populations as has been proposed by Garrison et al. [17].

3.8.2. Excited state conformational changes

The observed biexponential decay in viscous solvent systems and membranes has been attributed to emission from two different conformers, one with perpendicular orientation of the aromatic ring relative to the ester group (S_1^{FC}), relaxing to a second emitting state with more coplanar orientation. In order to verify this proposed intramolecular process, we performed semiempirical calculations on the model system 9-methylanthroate (Fig. 7c). As this compound does not contain the carboxyl headgroup present in the *n*-AS dyes, no intramolecular hydrogen bond can be formed and thus, only the geometric relationship between the ester function and the aromatic ring can influence the energy of the electronic states.

Table 3
Calculated heats of formation H_f and corresponding dihedral angles (°) for the local minima and transition states for the 2-AS model compound 2-(9-anthroyloxy)propionic acid

		Minimum 1	Transition state	Minimum 2
Hydrogen bond				
S_0	H_f (kcal/mol)	−102.56	−102.23	−102.33
	4-3-2-1 (°)	116°	78°	60°
S_1	H_f (kcal/mol)	−30.75	−29.70	−30.62
	4-3-2-1 (°)	138°	82°	44°
No hydrogen bond				
S_0	H_f (kcal/mol)		−106.45	
	4-3-2-1 (°)		120°	
S_1	H_f (kcal/mol)		−34.13	
	4-3-2-1 (°)		130°	

The transition state from a hydrogen bonded to a non-hydrogen bonded form was found at -97.67 kcal/mol.

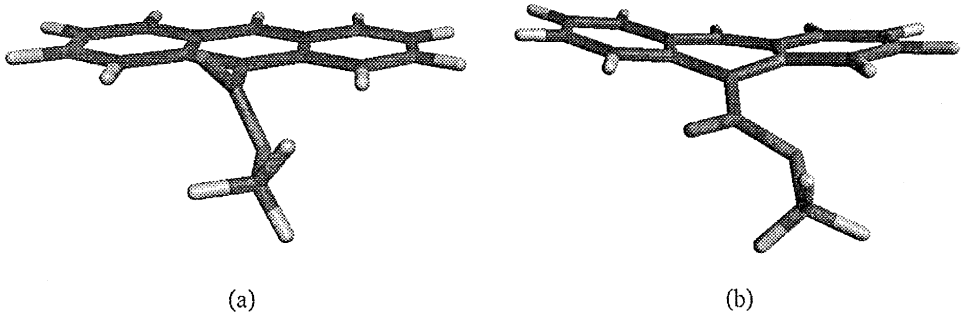

Fig. 8. AM1 optimized geometries of 9-methylanthroate in the electronic ground state (a) and the relaxed first excited singlet state (b).

All geometries were optimized using the AM1 hamiltonian, the excited states including a configuration interaction with 24 active orbitals. As reaction coordinate, the dihedral angle (4-3-2-1) between the COOMe group and the aromate was used (Fig. 7c). Fig. 8 shows the optimized conformations of the model ester in the electronic ground state (a) and first excited singlet state (b), respectively. It can be seen that a dihedral angle (4-3-2-1) of $\approx 60°$ between the plane of the anthracene system and the ester group is favoured in the ground state, while an angle of 0° was found to be most unfavourable due to steric repulsion of the peri-hydrogens of the aromate. Upon vertical excitation, the geometry of the system is not changed. This corresponds to a Franck–Condon transition into the S_1^{FC} state, which relaxes to a more planar form S_1^{EQ}. The dihedral angle decreases from 60° to 16° during the optimization, proving that a more planar orientation is favoured in the relaxed excited singlet state. Switching back to the electronic ground state, the vibrationally excited molecule S_0' relaxes to finally reach the starting geometry S_0. Therefore, calculations clearly indicate an intramolecular rotation following the electronic excitation for this 9-substituted anthracene compound.

4. Discussion

4.1. Decay behaviour

The fluorescence decay behaviour of the *n*-AS dyes has been studied extensively both in solvent systems and liposomes or membranes [4–7,17,19,33]. In isotropic fluid solvents the decay behaviour has generally been found to be monoexponential and independent of the site of attachment of the fluorophore. Our results in the non-polar solvent 3-methylpentane are in agreement with data for the series of *n*-AS in hexane [36], if the decays are detected at the emission maximum. However, a dependence of the decay behaviour on the emission wavelength was detected at the blue edge of the emission spectrum, where the decay is biexponential. Thus, some contribution from unrelaxed states at the shortest emission wavelengths may be assumed.

In contrast, the decay behaviour of 2-AS was completely independent of both excitation and emission wavelength in ethanol. The decay times for 6-, 9- and 12-AS are in agreement with recent data obtained by the phase modulation technique [17]. However, we found neither evidence for a biexponential decay in the case of 2-AS, as reported by Garrison et al. [17], nor for 3-AS, exhibiting a lifetime value of 5.8 ns compared to 7.6 ns for 2-AS and ≈ 3.0 ns for all other *n*-AS. The two lifetimes found by these authors have been assigned to two distinct populations of the excited state for 2-AS in ethanol due to an intramolecular hydrogen bonded state and an intermolecular hydrogen bonded state with the solvent [17].

Since our experimental results contradict this two state model we have performed semiempirical calculations on a model compound for 2-AS (2-(9-anthroyloxy)propionic acid). The optimization of the geometries for the ground and the first excited singlet state shows that structures leading to intramolecular hydrogen bonding are not energetically favoured in the gas phase (Table 3). In addition, the barrier between a hydrogen bonded state and the state with-

out hydrogen bond of about 9 kcal/mol seems to be too small to allow detection of individual states. If any intramolecular hydrogen bonding was important, this should be observed in an unpolar solvent or in the gas phase, where no other species for intermolecular stabilization are available. Therefore, intramolecular hydrogen bonding should not play any role in the polar protic solvent EtOH. Possible other reasons for the longer decay time of 2-AS and 3-AS are currently investigated by further calculations.

The decay behaviour in paraffin oil has been found to be complex and strongly dependent on emission wavelength, in agreement with recent work of Berberan–Santos and Prieto [19]. The observation of a negative pre-exponential factor is typical for the occurrence of an excited state reaction.

In most studies using *n*-AS in membrane systems fluorescence decays were only obtained at a single emission wavelength, typically near the emission maximum [21]. As the fluorescence decay does not deviate too much from monoexponential in this wavelength region the real complexity of the decay has not been realized in many early studies. The wavelength dependence of the decay times was first reported by Matayoshi and Kleinfeld for 2- and 12-AS [5]. We have shown that the absolute increase in the average decay times from 390 to 530 nm is similar for 2-, 6-, 9- and 12-AS, but considerably smaller for 16-AP. The percentage increase is largest for 2-AS which is located nearest to the lipid/water interface. The decay at the blue edge of the emission spectrum could best be fitted by a three-exponential model. This shows the heterogeneity of the decay but does not necessarily mean that only three distinct states contribute to the decay. Rather, it seems reasonable to assume that a continuum of states, either with different orientation of the anthracene moiety relative to the carbonyl group or with different solvent stabilization, contribute to the complex decay at short emission wavelengths.

4.2. Time-resolved emission spectra

Wavelength-dependent decays can show the existence of an excited state reaction (if ground state heterogeneity may be excluded) but cannot distinguish between a two-state model or a continuous relaxation process. This may be accomplished, however, if TRES are available, monitoring the fluorescence as a function of time after excitation.

A two-state model has been proposed by Matayoshi and Kleinfeld with an initially excited Franck–Condon state with perpendicular orientation of the anthracene moiety relative to the carbonyl group, relaxing to a more coplanar equilibrium state. This intramolecular relaxation was assumed to cause the wavelength dependent decay. In fluid solvents this process will be rapid with no time-dependent Stokes' shift observable. While this behaviour was found in EtOH, a small spectral shift with time remained in the unpolar 3-methylpentane, suggesting some influence of the polarity of the solvent. In viscous environment, both intra- and intermolecular relaxation processes are slowed down. Thus, larger Stokes' shifts with time after excitation are observed in paraffin oil. The Stokes' shifts are essentially independent of the position of the fluorophore, as might be expected for an isotropic solvent. The reason for the slightly different behaviour of 2-AS is not clear but it could be related to the proximity of the ester- and the carboxyl group in this molecule. No polar contribution to the shift is expected for paraffin oil. Thus, the observed shifts of 7–10 nm may be attributed to the intramolecular relaxation into a more planar orientation of the aromatic ring relative to the carboxyl group [5], as we confirmed by semiempirical calculations on the 9-methylanthroate model system (Fig. 7c). A decrease in the dihedral angle from 60° to 16° was observed (Fig. 8), showing that a more planar orientation is favoured in the excited state.

In phospholipid vesicles a large increase of the shift in the series 16-AP < 12-AS < 9-AS < 6-AS < 2-AS has been observed, with a shift of 40 nm for 2-AS within 20 ns after excitation. The relaxation process slows down with increasing depth of incorporation of the fluorophore, as seen in the correlation functions (Fig. 6). Considering only the intramolecular relaxation process, these observations are difficult to explain. An intramolecular relaxation should mainly depend on the viscosity of the environment. As has been shown by steady-state anisotropy measurements [6,31,37] and other techniques the 'microviscosity' decreases from the headgroup region to the terminal methyl groups of the phospholipid bilayers. Therefore, the intramolecular relaxation should become faster going deeper into the bilayer. However,

an opposite trend for the mean solvent relaxation times is observed experimentally. Thus, a simple intramolecular relaxation model is not sufficient to describe the behaviour of the *n*-AS in phospholipid bilayers.

Time-dependent Stokes' shifts of polarity sensitive probes like Laurodan [38] and Prodan [34,39–41] in lipid bilayers have been attributed to solvent relaxation processes. The model of dipolar relaxation assumes that the spectral shift occurs after excitation and it is due to the interaction between the excited state dipole and the surrounding solvent dipoles. The total energy released in the relaxation process (causing the observed red shift) depends on both the dipole moment of the solute and the dielectric constant of the solvent [42,43]. Several studies using the series of *n*-AS have established the idea of a polarity gradient along the membrane normal [6,9,44]. From the early work of Werner et al. [13–15] it is known that this group of fluorophores shows a considerable increase in dipole moment due to excitation. Thus, it seems reasonable to assume that the large increase in the Stokes' shift in the series 16-AP to 2-AS occurs due to increased contributions of dipolar relaxation processes of the hydrated headgroups or water molecules dissolved in the bilayer, respectively. This is a multistep process, causing the observed continuous spectral shift. The relaxation is fastest for 2-AS which is located closest to the membrane/water interface and slows down with increasing depth of dye incorporation. For 16-AP, only a small shift is observable which may be entirely due to the intramolecular relaxation. While dominating the relaxation behaviour in non-polar viscous media we conclude that the intramolecular relaxation plays only a minor role in phospholipid bilayer systems. In the latter systems the continuous model of dipolar relaxation is more adequate to describe the position dependent behaviour of the spectral shifts.

Due to their defined localization within lipid bilayers the series of *n*-AS provides the possibility to study influences on the relaxation behaviour in different depth of a bilayer. In a further set of experiments we studied the influence of the PC/PS molar ratio and of the addition of calcium ions on the relaxation behaviour of 6- and 12-AS. The system PC/PS/Ca^{2+} has attracted much attention both in relation to membrane fusion [45,46] and to the binding of extrinsic membrane proteins like prothrombin which is involved in a key step of the blood coagulation cascade [47,48]. Similar to recent results using the polarity sensitive probes Prodan and Patman [34] the mean relaxation times of 6- and even 12-AS were sensitive to the molar content of PS and to the presence of Ca^{2+}.

As might be expected, the sensitivity was greater for 6-AS showing much larger percentual increases both in absence or presence of calcium ions than 12-AS (see Table 2). The results indicate a tighter phospholipid headgroup packing with increasing PS content and suggest a bridging of PS molecules by Ca^{2+} within the plane of the membrane leading to a decrease in lipid mobility. The increase in rigidity of the membrane and/or the decrease of polarity due to the presence of PS and Ca^{2+}, respectively, diminishes on approaching the bilayer center. Therefore, the relaxation behaviour of the *n*-AS molecules seems to be a useful tool to study the influence, for example of protein binding, on the membrane dynamics at different depth within the bilayer. Following the approach employing Patman and Prodan [34], the characterization of the solvent relaxation behaviour in vesicles monitored by the set of *n*-AS dyes provides the possibility to investigate selectively the molecular mechanism of the protein interaction with different domains of the bilayer. For example, if only 2-AS and 3-AS were affected by the binding of an extrinsic protein to the membrane surface one can conclude that the protein does not penetrate significantly into the bilayer but is bound only electrostatically to the membrane surface. On the other hand, if changes in the solvent relaxation behaviour can still be detected deep inside the bilayer for 9-AS or even 12-AS it seems reasonable to assume that the bound protein shows hydrophobic interactions with the bilayer.

The influence of binding of prothrombin and its N-terminal fragment 1 as well as of factor X_a on the relaxation behaviour of the set of *n*-AS is currently investigated.

5. Conclusions

In this work we have investigated the time-resolved fluorescence behaviour of a series of *n*-AS both in isotropic solvents and in vesicle systems.

Semiempirical calculations support the idea of an intramolecular relaxation occurring in the excited state but they could not find any evidence for an intramolecular hydrogen bonded state for 2-AS. The decay behaviour has been shown to be wavelength-dependent and complex in viscous environments like paraffin oil and phospholipid bilayers, especially at the blue edge of the emission spectrum, where three exponentials were necessary to obtain good fits. Time-resolved emission spectra for the *n*-AS in vesicle systems have been reported for the first time. In contrast to the isotropic paraffin oil, the Stokes' shifts and the mean relaxation times are strongly dependent on the position of the fluorophore incorporated in vesicle systems. Thus, both intermolecular solvent relaxation processes and intramolecular relaxation have to be taken into account to explain the time-dependent emission behaviour. Due to their well defined localization and sensitivity to membrane dynamics and polarity, the *n*-AS dyes seem to be very promising probes for the study of lipid–protein interactions.

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