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ORIGINAL PAPER

The New Fluorescent Membrane Probe Ahba: A Comparative Study with the Largely Used Laurdan

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Abstract Lipid bilayers have been largely used as model systems for biological membranes. Hence, their structures, and alterations caused on them by biological active molecules, have been the subject of many studies. Accordingly, fluorescent probes incorporated into lipid bilayers have been extensively used for characterizing lipid bilayer fluidity and/or polarity. However, for the proper analysis of the alterations undergone by a membrane, a comprehensive knowledge of the fluorescent properties of the probe is fundamental. Therefore, the present work compares fluorescent properties of a relative new fluorescent membrane probe, 2-amino-N-hexadecyl-benzamide (Ahba), with the largely used probe 6-dodecanoyl-N,N-dimethyl-2-naphthylamine (Laurdan), using both static and time resolved fluorescence. Both Ahba and Laurdan have the fluorescent moiety close to the bilayer surface; Ahba has a rather small fluorescent moiety, which was shown to be very sensitive to the bilayer surface pH. The main goal was to point out the fluorescent properties of each probe that are most sensitive to structural alterations on a lipid bilayer. The two probes were incorporated into bilayers of the well-studied zwitterionic lipid dimyristoyl phosphatidylcholine (DMPC), which exhibits a gel-fluid transition around 23 °C. The system was monitored between 5 and 50 °C, hence allowing the study of the two different lipid structures, the gel and fluid bilayer phases, and the transition between them. As it is known, the fluorescent emission spectrum of Laurdan is highly sensitive to the bilayer gel-fluid transition, whereas the Ahba fluorescence spectrum was found to be insensitive to changes in bilayer structure and polarity, which are known to happen at the gel-fluid transition. However, both probes monitor the bilayer gel-fluid transition through fluorescence anisotropy measurements. With time-resolved fluorescence, it was possible to show that bilayer structural variations can be monitored by Laurdan excited state lifetimes changes, whereas Ahba lifetimes were found to be insensitive to bilayer structural modifications. Through anisotropy time decay measurements, both probes could monitor structural bilayer changes, but the limiting anisotropy was found to be a better parameter than the rotational correlation time. It is interesting to have in mind that the relatively small fluorophore of Ahba (o-Abz) could possibly be bound to a phospholipid hydrocarbon chain, not disturbing much the bilayer packing and being a sensitive probe for the bilayer core.

Keywords Ahba · Laurdan · DMPC · Steady state fluorescence · Time-resolved fluorescence

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Introduction

In the last decades, an extensive effort has been made to study cellular membrane properties and components. Lipids are the main component of cellular membranes, and lipid bilayers have been used as models to mimic these systems. In biophysics, several spectroscopic techniques are used to study the structure of lipid bilayers, such as fluorescence, nuclear and electronic magnetic resonance, light scattering, X-ray scattering, and others [1–8]. Among these techniques, fluorescence has several

advantages. First: due to its high sensitivity, small amounts of a fluorescent molecule can be detected. For systems that do not have intrinsic fluorescence, this is a huge advantage, because it is possible to use a tiny amount of extrinsic fluorescent molecules as probes, and the system under study is not altered by the presence of the probe. Second: the time scale of the fluorescent emission, in the nanoseconds range, is appropriated to detect structural dynamic properties of lipid bilayers. Third: fluorescent properties of a given probe intercalated into a membrane can change significantly due to the binding and/or penetration of a biomolecule into the membrane, therefore evidencing the structural alterations caused by that molecule. However, for the proper analysis of the alterations caused by a peptide, nucleotide, DNA or drug in a membrane, it is fundamental to know how sensitive the probe is to bilayer structural alterations.

The largely used fluorescent probe 6-dodecanoyl-N,N-dimethyl-2-naphthylamine (Laurdan), and analogues, were introduced as solvent-sensitive probes by Weber and Farris [9]. They were designed with electron donor and electron acceptor groups at opposite sides of a naphthalene ring, to induce the presence of a large electric dipole in the excited state. Laurdan has been widely used as a membrane probe, as its fluorescence spectrum is highly dependent on bilayer structure and/or polarity [2, 8, 10–23]. It anchors in the bilayer due to its 12-C aliphatic chain, with the fluorescent moiety localized close to the bilayer surface (Fig. 1).

More recently, 2-amino-N-hexadecyl-benzamide (Ahba) was introduced as a new membrane fluorescent probe [24]. This probe was synthesized as a lipophilic variation of the fluorescent probe o-Abz (ortho-aminobenzoic acid) used in the study of peptides, covalently attached to them [25–27].

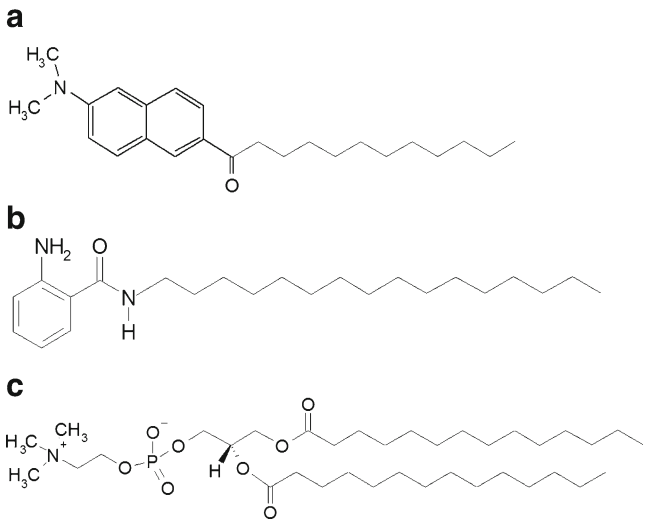

Fig. 1 The fluorescent probes, Laurdan (**a**) and Ahba (**b**), and the phospholipid DMPC (**c**)

In Ahba, the o-Abz group was bound to a long hydrocarbon chain, with 16 carbon atoms (Fig. 1), hence this probe also anchors at the bilayer, with the o-Abz group close to the membrane surface. Due to the small size of the fluorescent group o-Abz, Ahba becomes an interesting membrane probe, since it should scarcely disturb the lipid bilayer surface. However, the fluorescence spectrum of Ahba was found to be insensitive to the medium polarity, though the probe was found to be very effective in monitoring the pH value of charged surfaces of micelles and lipid vesicles [24].

The present work compares the sensitivity of several parameters obtained from static and time resolved fluorescence of Ahba and Laurdan incorporated into bilayers of the well-studied zwitterionic lipid dimyristoyl phosphatidylcholine (DMPC). This lipid exhibits a gel-fluid transition around 23 °C. The system was monitored between 5 and 50 °C, hence allowing the study of the two different lipid structures, the gel and fluid bilayer phases, and the transition between them.

Materials and Methods

Materials

DMPC (dimyristoyl phosphatidylcholine) was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL, USA). Laurdan (6-dodecanoyl-N,N-dimethyl-2-naphthylamine) was purchased from Molecular Probes Inc. (Eugene, OR, USA), Hepes (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid) and EDTA (ethylenediaminetetraacetic acid) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Milli-Q Plus water (Millipore), pH~6, was used for buffer preparation. Ahba (2-amino-N-hexadecyl-benzamide) was synthesized as described in [24].

Sample Preparation

A lipid film was formed from a solution of DMPC and the desired fluorescent probe (1 mol%) in chloroform. Chloroform was evaporated under a stream of N_2, and the sample left under reduced pressure for a minimum of 2 h, to remove all traces of organic solvent. Vesicles were prepared by the addition of 10 mM Hepes solution with 1 mM EDTA, pH 7.4, to a final lipid concentration of 1 mM. After hydration, the sample was vortexed for ~2 min and extruded through polycarbonate filters (21 times) with 100 nm pores, to yield large unilamelar vesicles. All procedures were carried out above the lipid phase transition temperature. The final lipid and probe concentrations were 1 mM and 10 μM, respectively.

Steady State Fluorescence Measurements

For steady state fluorescence spectroscopy, samples were placed in quartz cuvettes with 2 mm optical pathway. Measurements were performed using the VarianCary Eclipse. The temperature was controlled by a peltier system. Emission spectra were measured with excitation wavelength at 330 nm for Laurdan and Ahba. Steady state anisotropy data were obtained with the same equipment, using polarizers in the emission and excitation channels.

The steady-state anisotropy, r, is given by

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where I_{VV} and I_{VH} are the intensities with the excitation polarizer at the vertical position, and the emission polarizer at vertical and horizontal positions, respectively, and G is the ratio of the sensitivity of the system for vertically and horizontally polarized light [28]. The excitation wavelength was 330 nm for both probes and the emission wavelength was 405 nm for Ahba and 480 nm for Laurdan. For Ahba, 405 nm is close to the maximum fluorescence emission in DMPC bilayers (Fig. 2). For Laurdan, 480 nm is close to the maximum of one of the two components of its fluorescence spectrum [21]. This longer wavelength component is predominant at the fluid phase of the lipid, but it is present at all temperatures. At 480 nm, the Laurdan fluorescence emission was shown to be mostly due to this component only, at all temperatures [23].

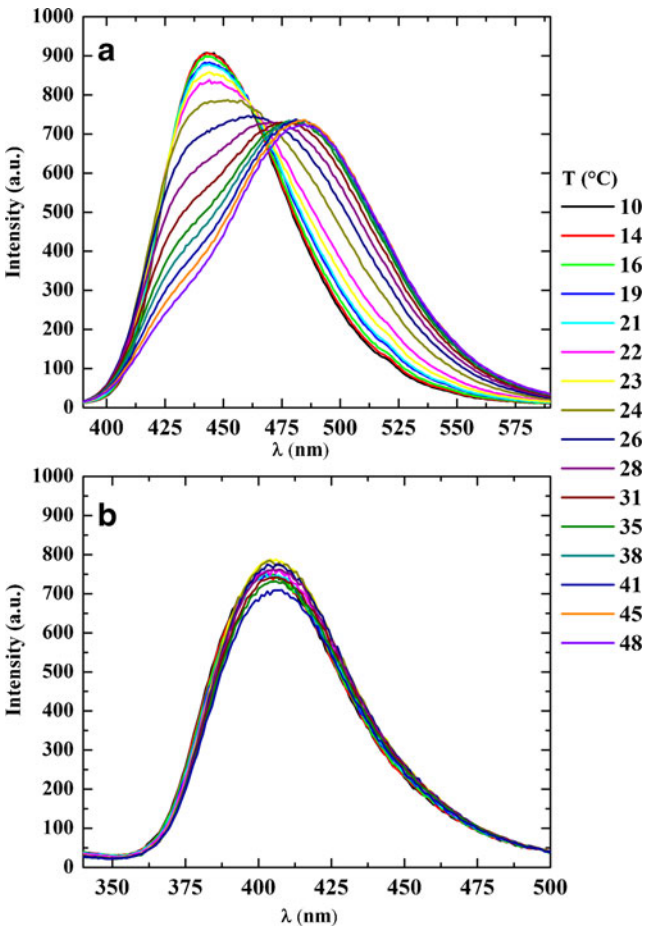

Fig. 2 Fluorescent emission spectra of Laurdan (**a**) and Ahba (**b**) in 1 mM DMPC aqueous dispersion, at different temperatures

Time-Resolved Fluorescence Measurements

Time-resolved fluorescence measurements were performed using the time-correlated single photon counting method (TCSPC). The excitation source used was a titanium-sapphire laser Tsunami 3950 from SpectraPhysics, pumped by a solid state laser Millenia Pro model J80, also from SpectraPhysics. The repetition rate was set to 8,000 kHz using a pulse picker (SpectraPhysics, model 3980-25). The Tsunami was set to give an output of 990 nm and a third harmonic generator BBO crystal (GWN-23PL SpectraPhysics) was used to generate the excitation beam at 330 nm. This beam was directed to a spectrofluorometer from Edinburgh FL900CDT. The emitted light was detected at 90° from the excitation beam. The emission wavelength was selected by a refrigerated microchannel plate photomultiplier (Hamamatsu R3809U). The FWHM of the instrument response function was 90–110 ps. Time resolution was 24 ps or 12 ps per channel in measurements for Ahba or Laurdan, respectively. The temperature control used was a Julabo HP 25.

A software package from Edinburgh Instruments was used to analyze the decay curves. The intensity decays were fitted to the equation:

$$I(t) = \sum_i \alpha_i e^{-\frac{t}{\tau_i}} \quad (2)$$

where τ_i is the lifetime of the i^{th} component of the decay, and α_i is the corresponding pre-exponential factor.

Fluorescence intensity decay data were analyzed both individually and globally. The two methods yielded very similar results. In the global analyses, at a fixed temperature, a set of intensity decay profiles, measured at different wavelengths, was considered (390, 400 and 410 nm for Ahba, and 470, 480 and 490 nm for Laurdan), and fitted with the same lifetime components, and different pre-exponential factors. The adequacy of the multiexponential decay fitting was evaluated by inspection of the residuals distribution and by the statistical parameter reduced chi-square (χ^2).

Fluorescence anisotropy decays were obtained through measurements of the fluorescence decay according to:

$$A(t) = \frac{I_{VV}(t) - GI_{VH}(t)}{I_{VV}(t) + 2GI_{VH}(t)} \quad (3)$$

where I_{VV}, I_{VH} and G are the same as in the steady-state

anisotropy but now measured as time decay intensities, using polarizers in front of the excitation and emission light. These fluorescence anisotropy decays were fitted to the following function:

$$F(t) = A_\infty + \sum_{i=1}^{n} \alpha_i e^{-\frac{t}{\phi_i}} \quad (4)$$

where α_i is the anisotropy at $t = 0$, immediately following the excitation pulse; ϕ_i is the rotational correlation time and A_∞ is called the limiting anisotropy. A_∞ is interpreted as resulting from an energy barrier that prevents rotational diffusion of the fluorophore beyond a certain angle [28]. The excitation wavelength was the same used in the intensity decay, 330 nm, and emission wavelengths were 410 and 480 nm, for Ahba and Laurdan, respectively.

Data shown in Figs. 3, 5 and 7 are means of three experiments, and the uncertainties are the standard deviations. When not shown, uncertainties were found to be smaller than the size of the symbols.

Results and Discussion

Steady State Fluorescence

Figure 2 shows the emission spectra of Laurdan and Ahba incorporated in 1.0 mM DMPC vesicles at different temperatures. Considering that DMPC has a gel-fluid transition around 23 °C, from 10 to 48 °C DMPC bilayer changes from a highly packed and ordered environment to a much more fluid, disordered, and more hydrated medium [29]. As largely reported [10–12, 14, 17, 18, 20, 21, 23, 30–33], Laurdan fluorescence spectrum (Fig. 2a) is extremely sensitive to bilayer structural alterations: the fluorescent emission band drastically changes from the gel to the fluid phase of the lipid. Laurdan spectrum can be decomposed into two components [21, 23], which are supposed to be related to two different excited probe populations, one more solvent relaxed than the other. The longer wavelength, attributed to the fluorescence emission from a more relaxed Laurdan excited state, would be favored by a looser and/or more hydrated micro-environment [17, 21, 23], hence predominant at higher temperatures.

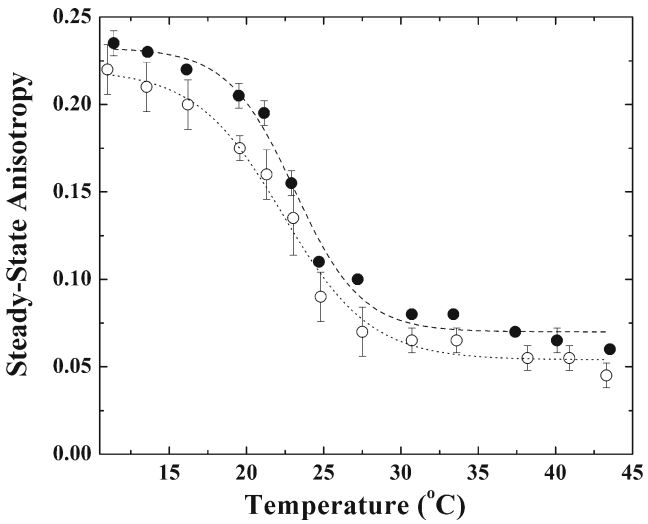

Fig. 3 Temperature dependence of the steady-state anisotropy of Ahba (○) and Laurdan (●) in 1 mM DMPC aqueous dispersion. Excitation wavelength was 330 nm, and emission wavelength was 405 nm for Ahba and 480 nm for Laurdan. Data points are linked by sigmoid curves

On the other hand, the fluorescent emission spectrum of Ahba (Fig. 2b) was found to be nearly unaffected by bilayer changes, either fluidity or polarity, as the spectrum is not sensitive to the bilayer temperature. Hence, the fluorescence emission spectrum of the probe Ahba should not be used in the analysis of alterations caused on the membrane structure (by the binding of drugs, for instance).

Opposite to that, and similar to Laurdan, the steady state fluorescence anisotropy of Ahba (Eq. 1) is extremely sensitive to changes in bilayer packing (Fig. 3). Sigmoidal curves were fitted to experimental data and the inflection points give DMPC phase transition temperature: 22.3±0.7 °C from Ahba anisotropy and 23.1±0.3 °C from Laurdan anisotropy data. These results are in good agreement with those reported in the literature [34, 35].

In Fig. 3, it is possible to observe that Laurdan yields higher fluorescence anisotropy values than Ahba, this could be an indication that Ahba is in a shallower position in the membrane, hence monitoring a more fluid environment. However, the difference between the two probes fluorescence anisotropy in DMPC membrane can be due to their different sizes. In the same microenvironment, the smaller Ahba could rotate faster than the bulky Laurdan, resulting in a higher light depolarization. Indeed, in ethanol, where the two probes are monomeric, Ahba (10 μM) fluorescence anisotropy was found to be 0.008 ±0.001 at 23 °C, comparing with that yielded by Laurdan at the same concentration and temperature, 0.020±0.005. Hence, it is possible to observe that Laurdan has a lower rotational mobility compared to Ahba. The fluorescence anisotropy of Ahba can be a good parameter to analyze the changes in lipid bilayer structure close to the surface.

Ahba is quite a small probe compared to other membrane probes, making it a particularly interesting probe, as its small size should not disturb the structure of the lipid bilayer, and its rotational movements could reflect more accurately the micro-environment viscosity.

Time Resolved Fluorescence

In many systems, time resolved fluorescence can bring significant information about structure and fluidity that are

not obtained with the steady state technique. Fluorescence intensity decays can identify the different conformational excited states of a macromolecule, and different excited states of a probe can be separately analyzed. Moreover, fluorescence anisotropy decay can provide information about the shape and flexibility of a molecule [28, 36]. Therefore, the sensitivity of time resolved fluorescence parameters yielded by the two probes, Ahba and Laurdan, were compared when they were incorporated in DMPC at different temperatures. Fluorescence intensity decay profiles were analyzed as illustrated in Fig. 4, taking into consideration the standard deviation (SD). As discussed in Materials and Methods, at each temperature, fluorescence intensity decays were analyzed by the global analyses, at three different emission wavelengths, 390, 400 and 410 nm for Ahba, and 470, 480 and 490 nm for Laurdan.

Ahba fluorescence decay profiles were best fitted with three exponentials (Eq. 2). The origin of these three lifetimes found for Ahba in DMPC is still a matter of investigation. Certainly, they are due to rather stable different excited states, and/or molecule structures, as they do not dependent on the medium polarity and viscosity, and dependent on the Ahba state of ionization only [24]. Figure 5a shows the temperature variation of the two longer lifetime components (○, △). The contribution of the smallest lifetime (~1.5 ns) was found to be very small, around 1 % for all temperatures, so it is not shown in Fig. 5. The small decrease of the lifetimes with the increase in temperature is probably related to the increase in non-radiative decays [28, 36]. Hence, neither the excited state lifetime values (Fig. 5a) nor the percentage of each of them (Fig. 5b) significantly change with the drastic structural changes that happens at the DMPC bilayer surface upon the lipid gel-fluid transition.

However, for Laurdan, the values of the two lifetime decays (Fig. 5a, ● and ▲) and their relative percentage (Fig. 5b, ● and ▲) are very sensitive to DMPC gel-fluid transition . These two lifetime decays are related to the two fluorescent emission bands (Fig. 2), and have been attributed to emission from two different excited states, a non-solvent-relaxed and a solvent-relaxed state [21, 23, 30]. Accordingly, the two excited state decays are sensitive to the bilayer gel-fluid transition, and Laurdan lifetimes decrease in the bilayer fluid phase (Fig. 5a), probably due to Laurdan-water relaxation. Moreover, there is a clear interconversion between the two species at DMPC gel-fluid transition (Fig. 5b), with the expected predominance of the more relaxed species (longer lifetime) at the lipid fluid phase. Thus, Laurdan lifetimes, both their values and the

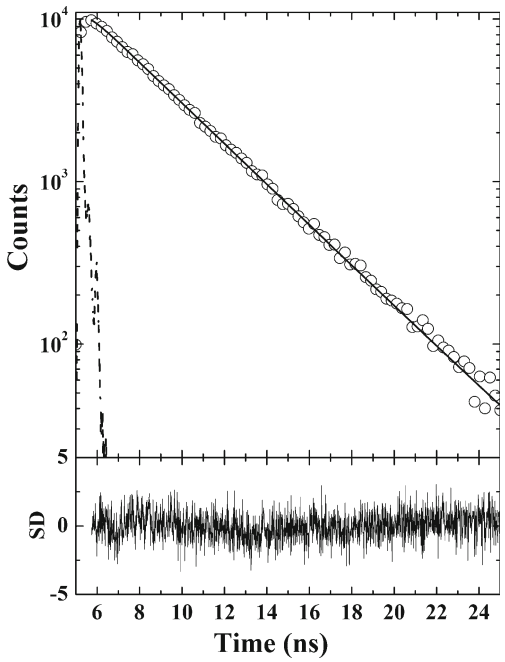

Fig. 4 Typical fitting (—) of the fluorescence intensity decay (○) of Laurdan in 1 mM DMPC aqueous dispersion (*dashed line* corresponds to the laser pulse). SD is the standard deviation. Some data points were removed from the graphic for better visualization

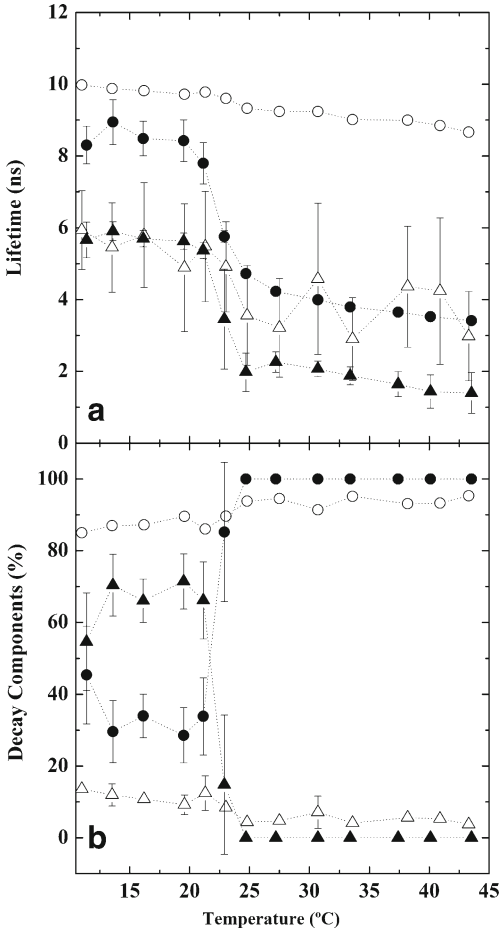

Fig. 5 Temperature dependence of lifetime values (**a**) and normalized pre-exponential factors (**b**) for the two longer lifetime components of Ahba (○, △) and the two lifetime components of Laurdan (●, ▲) in 1 mM DMPC aqueous dispersion

balance between them, are very sensitive to bilayer structure.

Results in Fig. 5b are in agreement with the fluorescence emission spectra measured for the two probes incorporated in DMPC (Fig. 2). Since the balance between the Ahba excited state populations does not change with temperature, the shape of its emission spectra also does not change when the lipid bilayer undergoes the phase transition. For Laurdan, the balance between the two excited state populations drastically changes at the lipid gel-fluid transition (Fig. 5b).

Time-resolved fluorescence anisotropy was analyzed as illustrated in Fig. 6. The anisotropy decay data were fitted to bi-exponential curves (Eq. 4), for both Ahba and Laurdan in 1 mM DMPC aqueous dispersion.

The longer rotational correlation time was well resolved for both probes, at all temperatures. Hence, Fig. 7a compares the temperature dependence of the longer rotational correlation time of Ahba and Laurdan. At the DMPC gel-fluid transition (23 °C), it is interesting to observe that no sharp change on rotational correlation times, of either Ahba or Laurdan, could be monitored, though Ahba correlation time seems to be highly dependent on the medium temperature, significantly decreasing as the temperature increases.

Another parameter that can be analyzed is the limiting anisotropy (A_∞ in Eq. 4), which monitors the fluorophore movement restriction. At times much longer than the rotational correlation time, if there is no restriction to the movement of the fluorophore, the molecule depolarizes all the emitted light, resulting in a limiting anisotropy equals to zero. However, for molecules whose movement can be restricted by the surrounding environment (a lipid bilayer, for example), the limiting anisotropy cannot reach zero, due to this restriction [28, 37, 38]. Interestingly, the limiting anisotropy (A_∞) clearly monitors a sharp decrease on the bilayer order at the DMPC transition temperature (23 °C, Fig. 7b). Presenting a larger A_∞ value, Laurdan in gel DMPC bilayer surface has its movements more restricted by the membrane than Ahba (larger A_∞ values in Fig. 7b). In fluid DMPC, no bilayer order is detected by the two probes (A_∞ around zero). Hence, for both Ahba and Laurdan, A_∞ seems to be a good parameter to be monitored for detecting alterations on the structure of a lipid membrane surface.

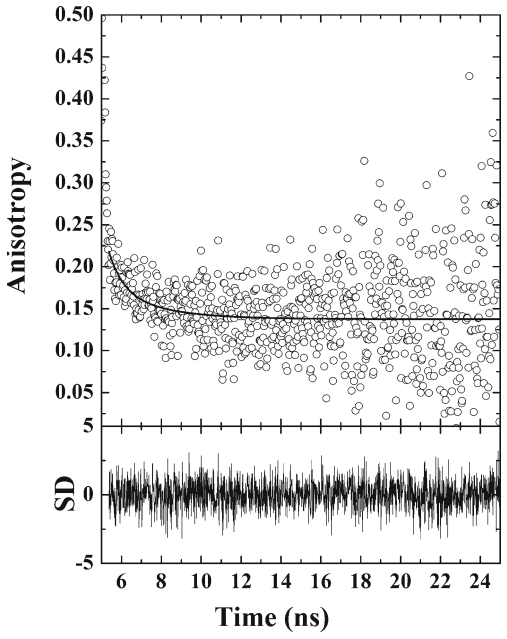

Fig. 6 Typical fitting (—) of the fluorescence anisotropy decay (○) of Laurdan in 1 mM DMPC aqueous dispersion. SD is the standard deviation. Some data points were removed from the graphic for better visualization

Conclusions

This work compares fluorescent properties of two probes, Ahba and Laurdan, which were synthesized to be intercalated into lipid aggregates, bilayers or micelles, with the fluorescent moiety close to the aggregate surface. The main

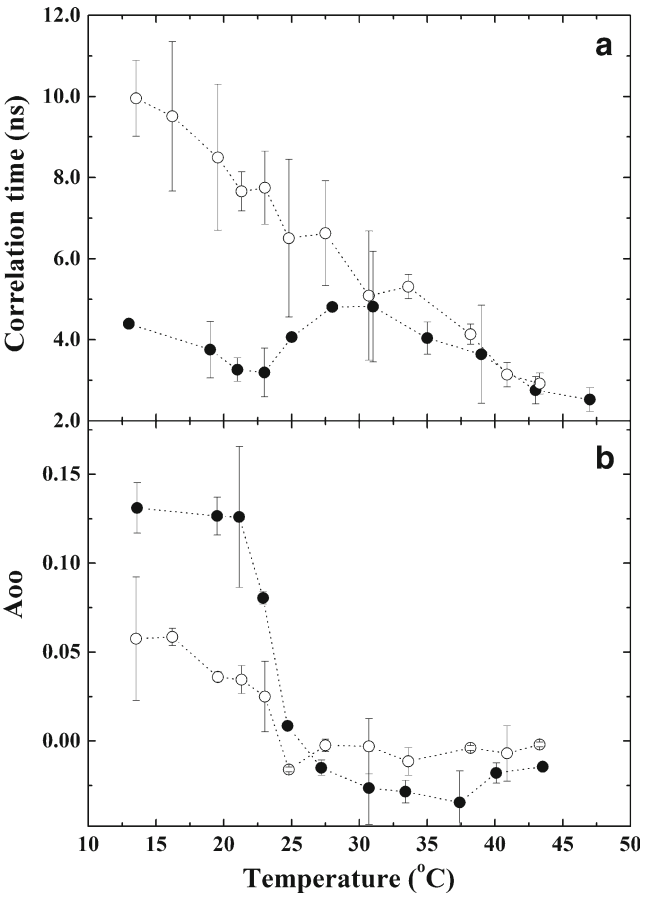

Fig. 7 Temperature dependence of longer rotational correlation times (**a**) and limiting anisotropy values, A_∞ (**b**), of Ahba (○) and Laurdan (●), in 1 mM DMPC aqueous dispersion

goal was to compare the new probe Ahba with the well-known Laurdan probe, and point out the fluorescent properties of each probe that are most sensitive to structural alterations on a lipid bilayer. Ahba is a relatively new fluorescent probe, with a convenient small fluorescent moiety, which has not been extensively analyzed. To enlighten the use of Ahba in lipid membranes, it is shown here that Ahba fluorescence intensity, and excited state lifetime decays, are not dependent on the medium polarity, viscosity or order (do not monitor a lipid gel-fluid transition). As expected [21, 39, 40], Laurdan fluorescent spectrum and excited state lifetimes were found to be very sensitive to DMPC bilayer structure. Steady state anisotropy measurements of both probes clearly monitor bilayer structural changes. Moreover, monitoring the fluorescence anisotropy decay, limiting anisotropy (A_∞) values of both Ahba and Laurdan were found to be good parameters to be used to monitor lipid structural modifications.

It is shown here that Ahba, like Laurdan, can be used to monitor bilayer structural changes, but the former only through fluorescence steady state and time resolved anisotropy data. It is interesting to have in mind that the relatively small fluorophore of Ahba (o-Abz) could possibly be bound to a phospholipid hydrocarbon chain, not disturbing much the bilayer packing and being a sensitive probe for the bilayer core.

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