On the Interaction of the Anthraquinone Barbaloin with Negatively Charged DMPG Bilayers

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Barbaloin is a bioactive glycosilated 1,8-dihydroxyanthraquinone present in several exudates from plants, such as *Aloe vera*, which are used for cosmetic or food purposes. It has been shown that barbaloin interacts with DMPG (dimyristoylphosphatidylglycerol) model membranes, altering the bilayer structure (Alves, D. S.; Pérez-Fons, L.; Estepa, A.; Micol, V. *Biochem. Pharm.* **2004**, *68*, 549). Considering that ESR (electron spin resonance) of spin labels is one of the best techniques to monitor structural properties at the molecular level, the alterations caused by the anthraquinone barbaloin on phospholipid bilayers will be discussed here via the ESR signal of phospholipid spin probes intercalated into the membranes. In DMPG at high ionic strength (10 mM Hepes pH 7.4 + 100 mM NaCl), a system that presents a gel−fluid transition around 23 °C, 20 mol % barbaloin turns the gel phase more rigid, does not alter much the fluid phase packing, but makes the lipid thermal transition less sharp. However, in a low-salt DMPG dispersion (10 mM Hepes pH 7.4 + 2 mM NaCl), which presents a rather complex gel−fluid thermal transition (Lamy-Freund, M. T.; Riske, K. A. *Chem. Phys. Lipids* **2003**, *122*, 19), barbaloin strongly affects bilayer structural properties, both in the gel and fluid phases, extending the transition region to much higher temperature values. The position of barbaloin in DMPG bilayers will be discussed on the basis of ESR results, in parallel with data from sample viscosity, DSC (differential scanning calorimetry), and SAXS (small-angle X-ray scattering).

Introduction

The gel−fluid thermal transition of the anionic lipid dimyristoylphosphatidylglycerol (DMPG), at high ionic strength, is rather cooperative, happening in a narrow temperature interval, around 23 °C, similar to that of the zwitterionic lipid dimyristoylphosphatidylcholine (DMPC) (Marsh, D. *Handbook of Lipid Bilayers*; CRC Press: Boston, 2000). However, at physiological pH but low ionic strength, DMPG presents a very interesting and unusual thermal profile related to the gel−fluid transition. It exhibits a rather complex differential scanning calorimetry (DSC) thermogram, with several calorimetric peaks between the beginning (T_m^{on} ∼ 17 °C) and the end (T_m^{off} ∼ 35 °C) of the melting process.[1−4] Although this transition region is not structurally well understood, some of its characteristics are known: low turbidity;[2,5,6] high viscosity and conductivity;[2,5] a Bragg peak in small-angle X-ray scattering (SAXS), at about 400 Å;[7] and a composite 16-PCSL electron spin resonance (ESR) spectrum, associated with the coexistence of rather fluid and hydrated domains, possibly high curvature regions, with patches that are more rigid and hydrophobic.[8] The transition region narrows significantly as the ionic strength increases, until a single main phase transition at T_m ∼ 23 °C is observed around 100 mM NaCl.[1,4] On the basis of the available data, it has been suggested that in the gel−fluid transition region DMPG could be structured as perforated vesicles,[7] which could evolve in tattered bilayer fragments.[9] Though it is well-established that no vesicle fusion takes place in DMPG low salt dispersions, at any temperature,[4,9] the presence of loose aggregates in the gel and fluid phases and their disaggregation in the gel−fluid transition region have been proposed[5,10] and cannot be ruled out.[9]

In studying the interaction of the anthraquinone barbaloin (10-glucopyranosyl-1,8-dihydroxy-3-(hydorxymethyl)-9(10*H*)-anthracenone, Aloin A) with DMPG membranes at high ionic strength (100 mM NaCl, close to the physiological condition), it was found that some of the attributes of the transition region of DMPG at low ionic strength were present, like a decrease in turbidity and a somewhat complex DSC profile.[11] Barbaloin is a known active ingredient extracted from leaves of different *Aloe* plants[12,13] and an effective herbal component traditionally used in China for treating various ailments.[14,15] For instance, it has the ability to diminish inflammation and infection[16] and has been widely used in light industry, especially in cosmetics and food products.[17]

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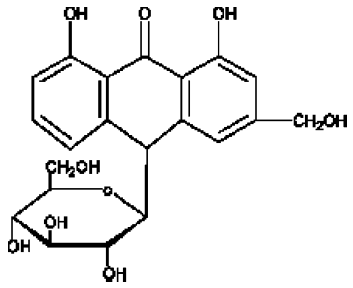
Scheme 1. Chemical Structure of the Anthraquinone Barbaloin



Considering the interesting results found with barbaloin in DMPG, and the wide pharmacological activity of this molecule, it was thought important to investigate if the anthraquinone would also elicit in high ionic DMPG other structural characteristics typical of the anomalous gel−fluid transition region observed at low ionic strength. It is important to have in mind that DMPG can be thought of as a model system of acidic lipid domains in natural membranes.

In addition to those studies, the structural alteration barbaloin causes in low ionic strength DMPG bilayers was also analyzed. This is far from physiological conditions, but it is an interesting physicochemical system that can give important information about the interactions present in an anionic bilayer.[1−10]

In this context, the effect of barbaloin on DMPG membranes was studied with electron spin resonance (ESR) of spin-labeled phospholipids intercalated into the bilayers, to monitor the structure of the gel and fluid phases, and the gel−fluid transition region. Three different lipid labels were used, with the paramagnetic moiety at the 16th, 12th, and 5th carbon atom (16-, 12-, 5-PCSL). In parallel to those measurements, viscosity and small-angle X-ray scattering (SAXS) data will be presented here.

Although barbaloin widens the gel−fluid thermal transition of high ionic strength DMPG bilayers, it does not seem to induce a transition region with the same peculiar characteristics found in low ionic strength DMPG dispersions. However, in those dispersions (low salt DMPG; 10 mM Hepes pH 7.4 + 2 mM NaCl), barbaloin strongly affects the bilayer properties, both in the gel and fluid phases. Surprisingly, 20 mol % barbaloin extends the DMPG gel−fluid transition region to temperatures above 60 °C. Hence, the present work not only discusses the interaction of barbaloin with a physiological relevant system, namely DMPG at high ionic strength, but also contributes to a better understanding of the peculiar structural characteristics of DMPG at low ionic strength.

Materials and Methods

Materials. The sodium salt of DMPG (1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol), cholesterol, and the spin labels 1-palmitoyl-2-(*n*-doxylstearoyl)-*sn*-glycero-3-phosphocholine (*n*-PCSL, $n = 5$, 12 and 16) were purchased from Avanti Polar Lipids (Birmingham, AL). Barbaloin (10-glucopyranosyl-1,8-dihydroxy-3-(hydorxymethyl)-9(10*H*)-anthracenone), Hepes (4-(2-hydroxyethyl)-1-piperizineethanesulfonic acid), and NaCl were purchased from Sigma Chemical Co. (St. Louis, MO). All reagents were used without further purification. Milli-Q water was used throughout.

Lipid Dispersion Preparation. A film was formed from a chloroform solution of DMPG, with barbaloin or cholesterol, when required. For ESR measurements, 0.2 mol %, relative to the lipid, for 16-PCSL, 0.4 mol % for 12-PCSL, and 0.6 mol % for 5-PCSL were added. Those were found to be the maximum spin label concentrations to display no spin−spin interaction. The chloroform solution was dried under a stream of N_2 and left under reduced pressure for a minimum of 2 h, to remove traces of the organic solvent. Dispersions were prepared with film hydration by the addition of the buffer solution, heated above the DMPG phase transition, at 40 °C for 2 min, and vortexed. The buffer systems used were 10 mM Hepes with 2 mM NaCl (low ionic strength) or 100 mM NaCl (high ionic strength), at pH 7.4. The total lipid concentration was 10 mM, or 50 mM for the SAXS experiments.

ESR Spectroscopy. ESR measurements were performed with a Bruker EMX spectrometer. The sample temperature was controlled within 0.1 °C by a Bruker BVT-2000 variable-temperature device and varied from 5 to 50 °C. To ensure thermal equilibrium, before each scan, the sample was left at the desired temperature for at least 10 min. The ESR data were acquired immediately after sample preparation. A field-modulation amplitude of 1 G and microwave power of 10 mW were used.

All data shown are means of the results of at least two experiments, and the uncertainties are the standard deviations. When not shown, the uncertainties are smaller than the size of the symbols.

The 16-PCSL spectral parameters, at high temperature, were found by fitting each line to a Gaussian−Lorentzian sum function[18] taking advantage of the fact that the sum function is an accurate representation of a Gaussian−Lorentzian convolution, the Voigt function.[19] The procedure separates the Gaussian and Lorentzian components of the spectral lines and locates the resonance fields of the three ESR lines to a precision of milligauss. Hence, it allows correct calculations of the rotational correlation times (related to the Lorentzian component only) and the isotropic hyperfine splitting (distance between lines). The corrected B and C parameters were calculated as described by Bales,[19] as a function of the three line heights, hence making the corrections for the contribution of nonresolved hyperfine splitting. Rotational correlation times, τ_B and τ_C, were calculated with both parameters and using the principal components of the g and hyperfine tensors of doxylpropane.[20] The hyperfine splitting, a_o, was taken to be one-half the difference in the resonance fields of the high- and low-field lines (fit by the computer program).

For the highly anisotropic spectra of 5-PCSL, the isotropic hyperfine splitting was calculated from the expression[21,22]

$$a_o = (1/3)(A_{||} + 2A_{\perp})$$

where $A_{||}$ ($=A_{max}$) is the maximum hyperfine splitting directly measured in the spectrum, and

$$A_{\perp} = A_{min} + 1.4\left[1 - \frac{A_{||} - A_{min}}{A_{zz} - (1/2)(A_{xx} + A_{yy})}\right]$$

where $2A_{min}$ is the measured inner hyperfine splitting and A_{xx}, A_{yy}, and A_{zz} are the principal values of the hyperfine tensor for doxylpropane.[20]

Effective order parameters, S_{eff}, were calculated from the expression

$$S_{eff} = \frac{A_{||} - A_{\perp}}{A_{zz} - (1/2)(A_{xx} + A_{yy})}\frac{a_o'}{a_o}$$

where $a_o' = (1/3)(A_{xx} + A_{yy} + A_{zz})$.

Differential Scanning Calorimetry. The calorimetric data were carried out in a Microcalorimeter Microcal MC-2, with a scan rate of 10 °C/h (similar profiles were obtained with 5 and 20 °C/h scans). Baseline subtractions and peak integrals were done using the Microcal

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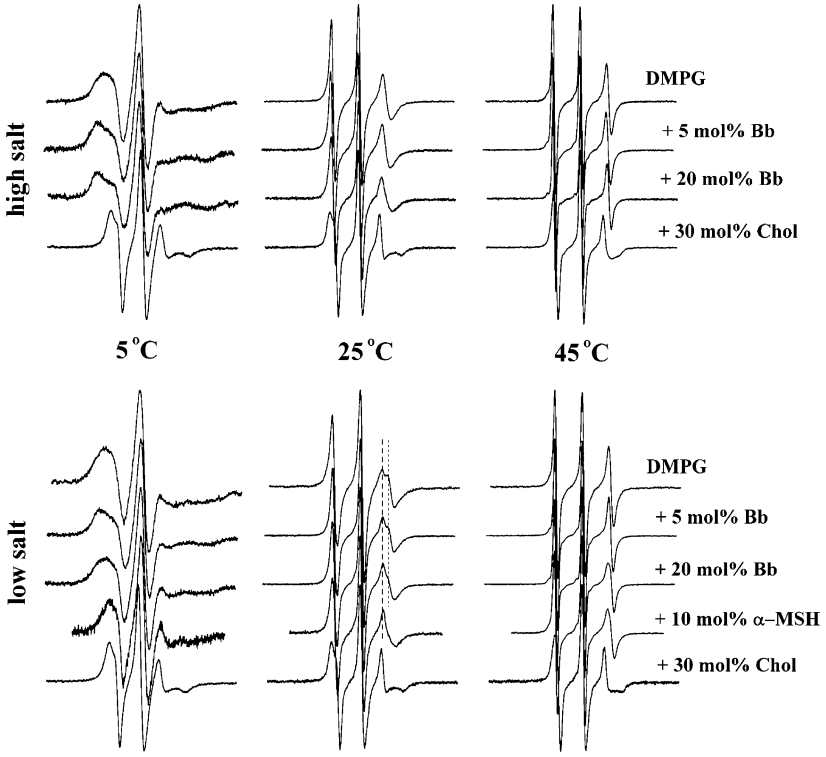

Figure 1. X-band ESR spectra of 0.2 mol % of 16-PCSL incorporated in 10 mM DMPG in Hepes buffer at high salt (100 mM NaCl) and low salt (2 mM NaCl). Temperatures and added molecules are indicated. The dashed and dotted lines make evident the two features at high-field position, in low salt DMPG, at 25 °C. α-MSH data are from previous work.[27]

Origin software with the additional device for DSC data analysis provided by Microcal.

Viscosity Measurements. Relative viscosities (ηsample/ηbuffer) were obtained using a ViscoClock Unit. A SCHOTT thermostat bath was used for temperature control. Temperature was varied from 5 to 50 °C. All data shown are means of the results of at least two experiments, and the uncertainties are the standard deviations.

Small-Angle X-ray Scattering Measurements. The measurements were performed at the D11A-SAXS beam line of the Brazilian Synchrotron Light Source (LNLS, Campinas, Brazil). The X-ray wavelength used was $\lambda = 1.608$ Å. The SAXS sample detector distance was 850 mm. Accurate intensities were measured in the range $0.008\ \text{Å}^{-1} \leq q \leq 0.350\ \text{Å}^{-1}$. A linear position sensitive detector was used, and a thermal bath was used for temperature variation (±0.2 °C). Samples were conditioned in a sample holder with 1-mm-thick spacer and Mylar windows. Data were normalized for the acquisition time (10−20 min), monitor integral counts (to compensate the variations in the beam intensity), sample attenuation, and DMPG concentration and corrected for the SAXS detector response (measured with a radioactive source). The measured scattering due to the buffer, which has no structure in the SAXS region, was subtracted from all scattering curves.

Results and Discussion

Structural Changes at the Bilayer Core. In Figure 1 the ESR spectra of 16-PCSL in pure DMPG bilayers, at low and high ionic strength, are compared with those recorded from the label in DMPG, with 5 and 20 mol % of barbaloin. For comparison, the spectra obtained with 30 mol % of cholesterol and 10 mol % of the cationic peptide α-melanotropic stimulating hormone (α-MSH; Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) are presented, the latter with DMPG low ionic strength only, as no effect is observed with DMPG at high ionic strength.[23] (We chose α-MSH because it is a hydrophilic molecule, which interacts at the bilayer surface, partially penetrating the membrane,[23,27] and has been extensively studied by one of us with DMPG, in the same buffer systems used here, with the same spin labels, so it is possible to compare the data.) The spectra are shown at three different temperatures, which, for pure DMPG, correspond to the gel (5 °C) and fluid (45 °C) phases and, for the low ionic strength sample, the gel−fluid transition region (25 °C).[4] Pure DMPG at high ionic strength has a sharp gel−fluid transition around 23 °C, so the bilayer is in the gel phase at 5 °C and in the fluid phase at 25 and 45 °C. As expected, the gel and fluid DMPG phases are well-characterized by a rather anisotropic and a much more isotropic ESR signal, respectively.

As mentioned before, the 16-PCSL ESR spectrum in low ionic strength DMPG gel−fluid transition region (at 25 °C in Figure 1) can be analyzed as the sum of two ESR signals.[8] The presence of the two signals can be clearly seen at the high field position of the ESR spectrum (see the lines in Figure 1, for pure DMPG at low salt, 25 °C). As extensively discussed,[8] there is a coexistence of rather fluid and fairly hydrated domains (the high field feature indicated by the dotted line in Figure 1) with patches more rigid and less hydrated (the high field feature indicated by the dashed line in Figure 1). As the two signals can be well-monitored between T_m^{on} and T_m^{off}, their presence has been used as one of the characteristics of the transition region of DMPG at low ionic strength.[8] The fact that two well-separated spectra are obtained shows that the exchange between spin labels in the two different membrane domains is slow on the ESR time scale ($\sim 10^{-9}$ s). Moreover, the ESR spectra yielded by 16-PCSL in the two domains is sufficiently different to come out separately.[8]

Figure 1 (at 25 °C) shows that the presence of barbaloin in DMPG bilayers at high ionic strength does not change the 16-PCSL ESR spectrum as to reproduce the spectrum yielded by DMPG at low ionic strength. Namely, barbaloin does not elicit in high ionic strength DMPG the 16-PCSL ESR double signal

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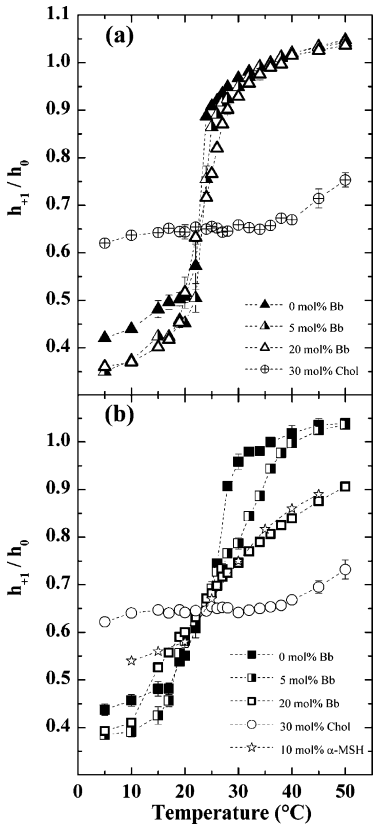

Figure 2. Temperature dependence of the ratio between the amplitudes of the low and central field resonance lines (h_{+1}/h_0), measured on the ESR spectra of 0.2 mol % of 16-PCSL incorporated in 10 mM DMPG in Hepes buffer at (a) high salt (100 mM NaCl) and (b) low salt (2 mM NaCl), pure and with barbaloin (Bb), cholesterol (Chol), and α-MSH.

obtained in low ionic strength lipid dispersion (see the lines in Figure 1 for pure DMPG at low salt, 25 °C). Hence, though barbaloin was found to alter the DSC and light-scattering profiles of DMPG at high ionic strength, making them somehow more similar to those of DMPG at low ionic strength,[11] this effect is not observed when the bilayer structure is monitored.

Interestingly, at low ionic strength DMPG bilayers, in the gel−fluid transition region (see Figure 1 at 25 °C), the presence of barbaloin changes the 16-PCSL ESR spectrum, decreasing the fraction of the more isotropic signal (indicated by the dotted line). This effect is different from that of the cationic peptide α-MSH, which completely destroys the more isotropic ESR signal (see Figure 1 at 25 °C and ref 8). Cholesterol, which penetrates the lipid bilayer, strongly changes the membrane structure, both at high and low ionic strength, as discussed below. At all temperatures, the 16-PCSL ESR spectrum in DMPG + cholesterol dispersions seems to be due to one signal only, hence indicating a homogeneous DMPG + cholesterol bilayer.

To better understand the structural alterations caused by barbaloin on DMPG bilayers, the 16-PCSL ESR spectra were analyzed through the ratio of the amplitudes of the central and low field lines (h_{+1}/h_0) (Figure 2), which tends to unity as the spin label mobility increases. This is the best parameter to be used with 16-PCSL over the whole range of temperature, from gel to fluid phase, to monitor the bilayer fluidity. However, this parameter must be treated with care, because if the spectrum is due to overlapping ESR signals, a changing in the mixture of the two can be confused with fluidity changes. Hence, between T_m^{on} and T_m^{off}, where the presence of the two peaks indicates the presence of two different ESR signals, the increase in h_{+1}/h_0 is most likely reflecting a mixture between changes in mobility and in the proportion of these two spin label populations.

For high ionic strength DMPG bilayers, where only one ESR signal seems to be present, making the spectrum analysis straightforward, barbaloin makes the phase transition somewhat less sharp or cooperative (Figure 2a), as was also seen by DSC.[11]

Interestingly, for low temperatures, in DMPG gel phase, 16-PCSL clearly monitors a much more packed region with barbaloin at low and high ionic strength, evident by the significant decrease of the h_{+1}/h_0 ratio. This is not the usual effect of a molecule that penetrates the membrane. For instance, cholesterol, which is known to penetrate the lipid bilayer, disturbing the highly organized gel lipid phase, yields the opposite effect, as shown in Figure 2a,b. (That can also be inferred from the more isotropic ESR spectra yielded by 16-PCSL in DMPG + cholesterol, at 5 °C in Figure 1). As expected, cholesterol broadens the thermal transition,[24] making it disappear as monitored by the 16-PCSL (Figure 2). Even the cationic peptide α-MSH, which probably resides at the bilayer surface, partially penetrating the hydrophobic core, disturbs the gel packing, increasing the ratio h_{+1}/h_0 (Figure 2b)

Barbaloin significantly alters the structure of low ionic strength DMPG bilayers. In addition to making the gel phase more rigid at 5 and 10 °C, at 15 °C the membrane is more fluid in the presence of 20 mol % of barbaloin than in pure DMPG. Moreover, the temperature range of the phase transition is considerably broadened, and the bilayer is much more packed for higher temperatures (lower h_{+1}/h_0 values in Figure 2b).

For a better analysis of the bilayer packing at the lipid fluid phase, rotational correlation times were calculated (see Materials and Methods). The two correlation times, τ_B and τ_C, were found to be rather similar above 40 °C, indicating a nearly isotropic movement for the probe,[25] in DMPG and DMPG + 20 mol % barbaloin (hence, only τ_C is shown in Figure 3a). For high ionic strength DMPG bilayers, barbaloin slightly increases the probe correlation times, indicating that it is somewhat increasing the bilayer packing (Figure 3a). In accord with the decrease in h_{+1}/h_0 ratios (Figure 2b), for low ionic strength DMPG, 20 mol % barbaloin considerably increases the correlation times, hence turning the DMPG bilayer significantly less fluid, though the effect is weaker than that observed with 10 mol % of the cationic peptide α-MSH.

For labels inside a lipid bilayer, there are strong indications that the presence of water in the bilayer can be estimated from the magnitude of the isotropic nitrogen hyperfine splitting a_o. The increase in a_o is ascribed to the increase in the spin label nitroxide−water H-bonds inside the bilayer.[26] In parallel with the small effect of barbaloin on the structure of the fluid phase of high ionic strength DMPG bilayer, the anthraquinone does not alter the membrane polarity (Figure 3b). Interestingly, the decrease in bilayer fluidity caused by barbaloin in fluid low ionic strength DMPG is correlated to a large increase in the isotropic hyperfine splitting (significant increase in 16-PCSL a_o), indicating an increase in membrane hydration at the bilayer core. The same effect was observed with the cationic peptide α-MSH, whereas cholesterol rigidifies the membrane, but considerably decreases its polarity.[27] Hence, the increase in bilayer packing associated with the increase in nitroxide−water H-bonds

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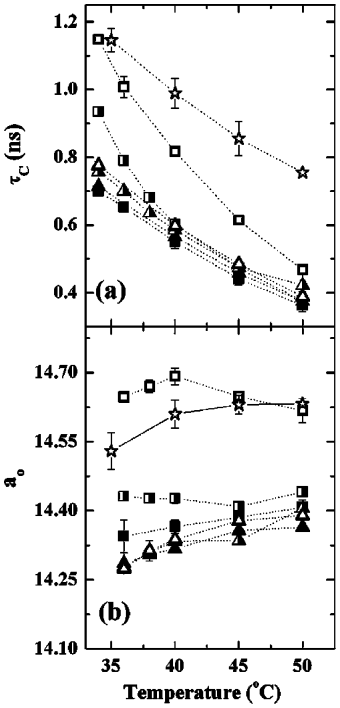

Figure 3. Temperature dependence of the (a) correlation time (τ_C) and (b) isotropic hyperfine splitting (a_o) measured on the ESR spectra of 0.2 mol % of 16-PCSL incorporated in 10 mM DMPG in Hepes buffer at low salt (2 mM NaCl), (■) in the absence of barbaloin (Bb) and the presence of (◨) 5 mol % and (□) 20 mol % of Bb and (☆) 10 mol % of α-MSH, and at high salt (100 mM NaCl), (▲) in the absence and presence of (◭) 5 mol % and (△) 20 mol % of Bb.

in the bilayer core seems to be a somewhat more general effect related to polar molecules interacting close to the bilayer surface and needs more investigation.

Structural Changes Close to the Bilayer Surface. Due to the flexibility gradient toward the bilayer core,[20] the spectra of 5-PCSL in DMPG membranes are much more anisotropic (larger A_{max}) than those of 16-PCSL, which monitor the center of the membrane. At the fifth carbon atom position, the changes caused by barbaloin on the DMPG bilayer structure are not obvious on the spectra (Figure 4). Over the whole range of temperatures, DMPG packing can be monitored through the variation of the parameter A_{max}, the outer hyperfine splitting (shown in Figure 5), measured on the 5-PCSL spectra, which can be used as an empirical parameter that increases with the label microenvironment viscosity or packing.[28] At the fifth carbon atom position, a highly packed/organized bilayer region, barbaloin does not seem to significantly change the gel phase of DMPG bilayers, at high and low salt dispersions. However, the broadening of the phase transition is evident, both at high and low ionic strength (Figure 5), the effect being stronger in the latter, as observed with 16-PCSL (Figure 2).

In the fluid phase, the bilayer can be better analyzed by the parameter S_{eff} (Figure 6). The main contribution to the effective order parameter, S_{eff}, is the amplitude of movement of the hydrocarbon chain moiety.[29] Barbaloin slightly increases the order of the fluid phase at the fifth C-atom position (increases S_{eff}) for both high and low ionic strength DMPG membranes, with a somewhat stronger effect for low ionic strength DMPG. However, similar to the effect observed at the bilayer core (Figure 3), barbaloin significantly increases the bilayer polarity of low ionic

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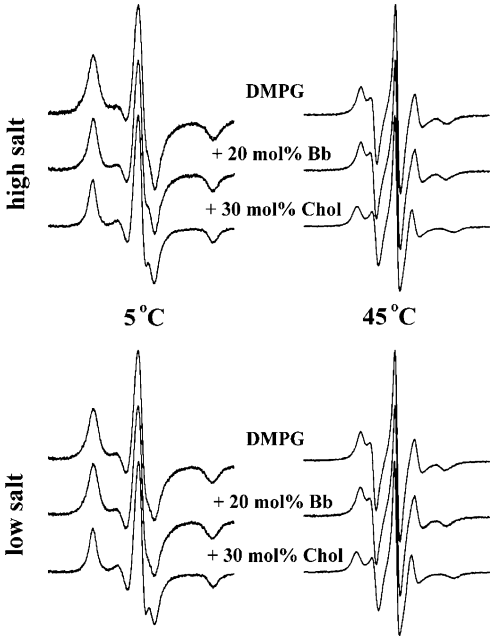

Figure 4. X-band ESR spectra of 0.6 mol % of 5-PCSL incorporated in 10 mM DMPG in Hepes buffer at high salt (100 mM NaCl) and low salt (2 mM NaCl). Temperatures and added molecules are indicated.

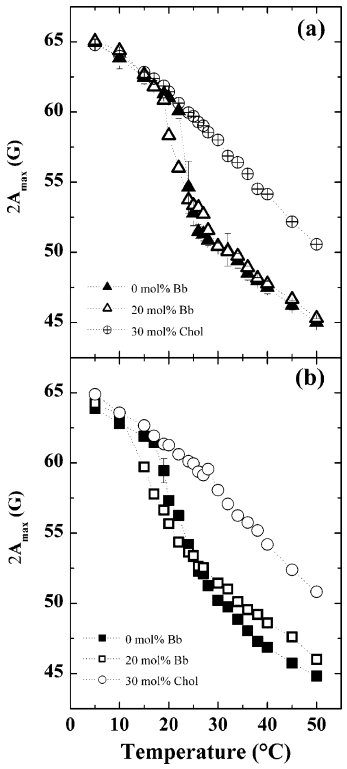

Figure 5. Temperature dependence of the outer hyperfine splitting (A_{max}) measured on ESR spectra of 0.6 mol % of 5-PCSL incorporated in 10 mM DMPG in Hepes buffer at (a) high salt (100 mM NaCl) and (b) low salt (2 mM NaCl).

DMPG bilayers, at the fifth C-atom position (see increase in a_o in Figure 6b). The effect of 20 mol % barbaloin is similar to that of 10 mol % of α-MSH and opposite of that of cholesterol.[27]

Structural Changes Close to the Bilayer Core. Though DMPG has 14 C-atom chains, it was shown that the paramagnetic center of 16-PCSL is localized at the DMPG bilayer core, yielding a rather isotropic signal, typical of a relatively mobile spin moiety.[27] However, the peculiar effect of barbaloin on increasing

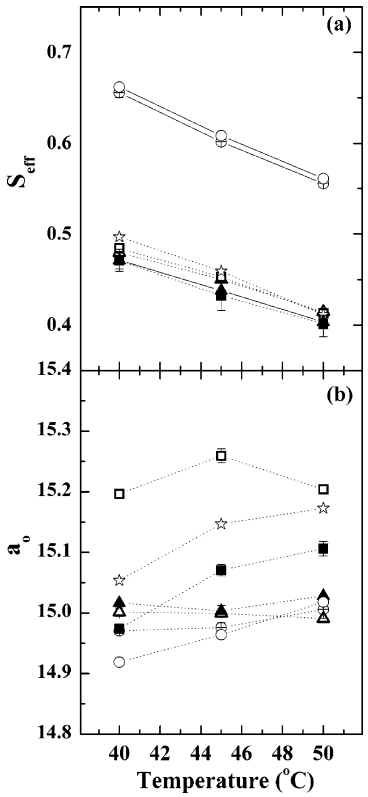

Figure 6. Temperature dependence of the (a) effective order parameter (S_{eff}) and (b) isotropic hyperfine splitting (a_o), measured on ESR spectra of 0.6 mol % of 5-PCSL incorporated in 10 mM DMPG in Hepes buffer at low salt (■) in the absence of barbaloin (Bb) and the presence of (□) 20 mol % Bb, (☆) 10 mol % α-MSH, and (○) 30 mol % of cholesterol (Chol) and at high salt (▲) in absence of Bb and the presence of (△) 20 mol % Bb and (⊕) 30 mol % of Chol.

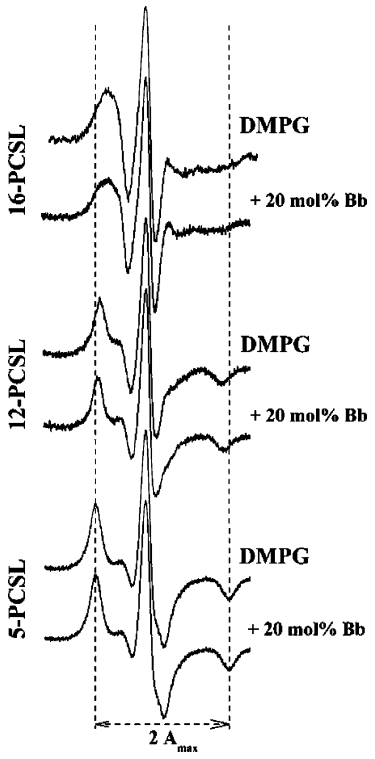

Figure 7. X-band ESR spectra of 16-, 12-, and 5-PCSL incorporated in 10 mM DMPG in Hepes buffer at low salt (2 mM NaCl) in the absence and presence of 20 mol % of barbaloin (Bb) at 5 °C. For comparison, the dashed lines indicate the maximum hyperfine splitting of the 5-PCSL spectrum.

so much the bilayer gel phase packing (Figure 2) could possibly be related to the anthraquinone somehow organizing the bilayer and forcing the spin label chain to penetrate into the adjacent layer, thus decreasing the movement of the 16-C atom of the labeled chain, which could be probing the adjacent layer around the 12th atom position. And/or barbaloin could be causing interdigitation in DMPG chains. Hence, to check these hypotheses, a phospholipid labeled at the 12th carbon atom of the chain, 12-PCSL, was incorporated into DMPG membranes, without and with 20 mol % barbaloin, and its ESR signal was compared with those of 16- and 5-PCSL. The ESR spectra of 16-, 12-, and 5-PCSL in DMPG gel bilayer, with and without barbaloin, at 5 °C, clearly indicate that the probes are monitoring the bilayer at different depths (Figure 7), with 16-PCSL displaying a much less anisotropic spectrum, due to its higher mobility at the bilayer core. So, no chain interdigitation is observed.[30] Moreover, the packing effect of barbaloin at the DMPG gel phase is also clearly monitored at the 12th C-atom position, as evident by the increase in the label parameter A_{max} (Figure 8). Hence, barbaloin increases the packing of the DMPG gel phase, both at high and low ionic strength, all over the hydrocarbon chain. Similar to the effect observed with 16-PCSL, in low ionic DMPG bilayers, barbaloin only increases the packing for temperatures below 15 °C.

With 12-PCSL, it was not possible to calculate a correlation time for the label in the DMPG fluid phase, as the spectra are not in the motional narrowing regime,[25] so the ratio between the amplitudes of the high and central field lines, h_{-1}/h_0, was measured. Like the h_{+1}/h_0 ratio, the h_{-1}/h_0 ratio tends to unity as the spin label mobility increases. Similar to the effect observed with 16-PCSL (Figures 2 and 3), barbaloin strongly rigidifies the DMPG fluid phase, mainly at low ionic strength (Figure 9).

At the lipid fluid phase, the three molecules, barbaloin, cholesterol, and the cationic peptide α-MSH, which certainly interact differently with DMPG, turn the bilayer more rigid and/or organized. That is evidenced by the increase in the 16-PCSL rotational correlation time τ_C (Figure 3a), the decrease in the 16- and 12-PCSL h_{-1}/h_0 ratio (ref 27 and Figure 9), and the small increase in the 5-PCSL effective order parameter (Figure 6a).[2] (The observed small effect at the fifth carbon atom position is expected, as this is a rather organized part of the bilayer, with the spin label ESR spectra at the limit of its sensitivity.) Considering that it is rather unlikely that either barbaloin or α-MSH would penetrate the bilayer to its core, it seems that a molecule that partially penetrates the lipid hydrocarbon chains, without separating them too much, is capable of increasing the order down to the bilayer center. That could be rationalized by having in mind that the mobility of a C−C moiety at the bilayer core is dependent on the mobility of the whole chain. So, even if the interacting molecule restrains the freedom of the first two or three carbons only, that could be propagated till the end of the hydrocarbon chain, reducing the overall chain configuration space.

Macroscopic Data. *DSC and Viscosity.* Figure 10a shows a typical DSC profile of 10 mM DMPG at low ionic strength. A pretransition is clearly observed at ∼11 °C, and we will not focus on this transition here. As discussed before,[7] the peculiar gel−fluid transition of low-ionic DMPG region starts at T_m^{on} = 18.2 °C and ends at T_m^{off} = 31.7 °C. Interestingly, the presence of even 5 mol % barbaloin significantly broadens the T_m^{on} peak, hence making the thermal event related to this peak less cooperative. Barbaloin decreases T_m^{on} (∼16.2 °C) and shifts T_m^{off} to a higher value, ∼36.2 °C. The higher barbaloin concentration,

(30) Bartucci, R.; Páli, T.; Marsh, D. *Biochemistry* **1993**, *32*, 274−281.

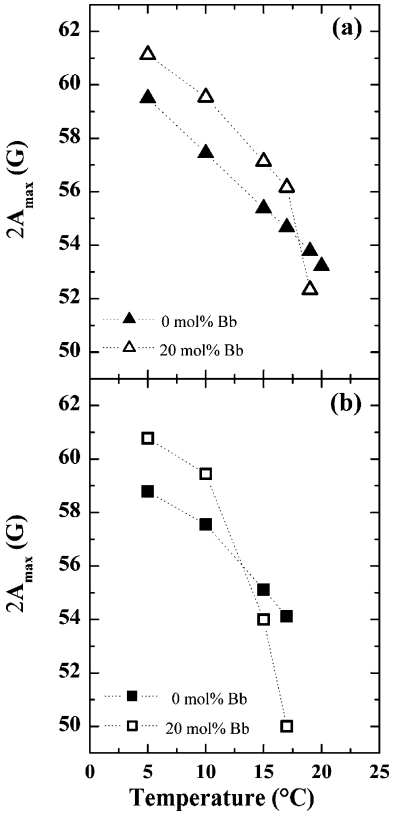

Figure 8. Temperature dependence of the outer hyperfine splitting (A_{max}) measured on the ESR spectra of 0.4 mol % of 12-PCSL incorporated in 10 mM DMPG in Hepes buffer at (a) high salt (100 mM NaCl) and (b) low salt (2 mM NaCl).

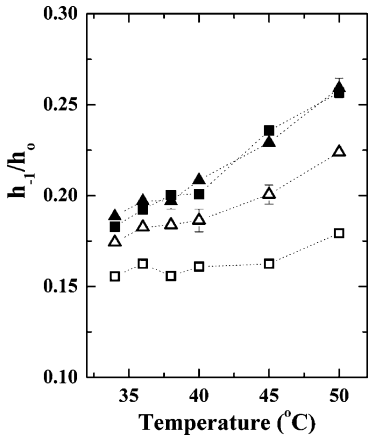

Figure 9. Temperature dependence of the ratio between the amplitudes of the high and central field resonance lines (h_{-1}/h_0) measured on the ESR spectra of 0.4 mol % of 12-PCSL incorporated in 10 mM DMPG in Hepes at low salt in (■) the absence of barbaloin (Bb) and the presence of (□) 20 mol % of Bb and at high salt in (▲) the absence of Bb and the presence of (△) 20 mol % of Bb.

20 mol %, nearly completely effaces T_m^{on} (~12.7 °C) and either makes T_m^{off} disappear or shifts it to temperatures higher than 50 °C.

Considering that the DMPG gel−fluid transition region has also been characterized by the high viscosity of the dispersion,[2] this macrocharacteristic of the lipid dispersion was measured in the presence of 5 and 20 mol % barbaloin (Figure 10b). In parallel with DSC measurements, 5 mol % barbaloin broadens the DMPG transition region monitored via its anomalous high viscosity. Interestingly, for 20 mol % barbaloin, the sample viscosity is rather high up to 50 °C, suggesting that barbaloin keeps DMPG

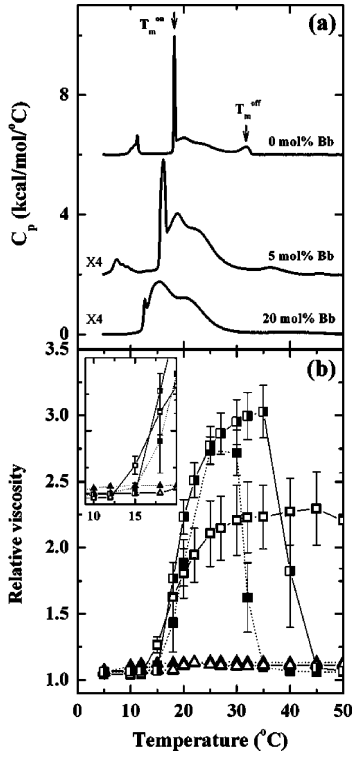

Figure 10. (a) Differential scanning calorimetry heating thermograms for 10 mM DMPG in Hepes buffer at low salt in the absence and presence of barbaloin (Bb). (b) Relative viscosity ($\eta_{sample}/\eta_{buffer}$) *versus* temperature of 10 mM DMPG in Hepes buffer at low salt in (■) absence of Bb and the presence of (◨) 5 mol % and (□) 20 mol % of Bb and at high salt in (▲) the absence of Bb and the presence of (▲) 5 mol % and (△) 20 mol % of Bb. The DSC curves are shifted for clarity. The DSC curves for DMPG in the presence of Bb were amplified (indicated on the left) to fit in the same scale as that of pure DMPG.

in the "peculiar" transition region at least up to 50 °C. This is in accord with the absence of T_m^{off} in the DSC scan shown in Figure 10a, as T_m^{off} signals the end of the transition region. Viscosity measurements also show that 20 mol % barbaloin decreases T_m^{on}, the beginning of the gel−fluid transition region (see the inset). This is in accord with ESR data (Figures 2b, 5b, 8b).

Though barbaloin was found to significantly broaden the DMPG DSC profile at high ionic strength,[11] no significant effect could be observed in the sample viscosity (Figure 10b).

SAXS Results. SAXS results with DMPG low and high ionic strength, with and without barbaloin, are presented here (Figure 11), though they will be better discussed elsewhere (Duarte et al., in preparation).

In a previous work,[3] DMPG dispersions were studied by SAXS. A broad peak was detected at all lipid phases, consistent with the expected scattering for large unilamellar vesicles. No Bragg peak characteristic of multilamellar vesicles was observed, in accord with charged lipids avoiding the formation of ordered multilamellas, due to electrostatic repulsion. The broad peak could be well-fitted by a temperature-dependent bilayer form factor.[3] However, in the low ionic strength DMPG gel−fluid transition region, by looking at very low angles [low q values, q being the scattering vector modulus given by $q = 4\pi \sin(\theta)/\lambda$, where 2θ is the scattering angle and λ is the wavelength], a new peak could be detected, corresponding to a repetition distance of about 400 Å.[7]

We want here to draw attention to this low q value peak only (see arrows in Figure 11a,b). This peak is a fingerprint of the

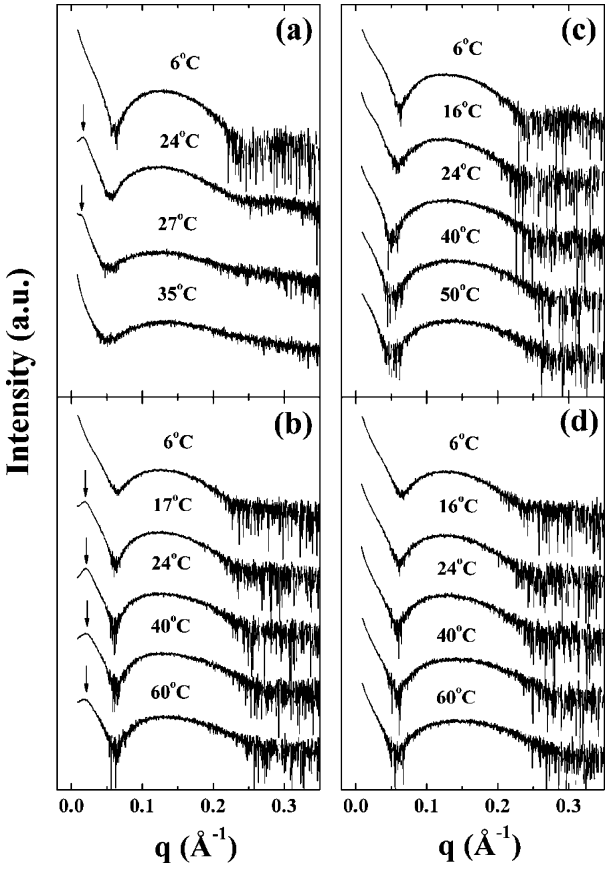

Figure 11. SAXS curves at indicated temperatures of 50 mM DMPG in Hepes buffer at low salt (2 mM NaCl) in (a) the absence and (b) the presence of 20 mol % of Bb and at high salt (100 mM NaCl) in (c) the absence and (d) the presence of 20 mol % of Bb.

DMPG anomalous temperature transition region and was proposed to arise due to in-plane correlated perforations.[7] Hence, it would be related to a repetition distance between lipid bilayer domains coexisting with membrane pores, the latter containing lipids in micelle-like structure. This hypothesis had the support of the ESR results, which made evident the existence of two populations with different mobility and polarity (evident in the 16-PCSL double peak at high magnetic field in Figure 1, and ref 8).

Figure 11d shows that barbaloin, in a high ionic strength DMPG dispersion, does not bring forth the low *q* value peak, typical of the low ionic strength DMPG transition region. This is in accord with barbaloin not eliciting the double peak at the 16-PCSL spectra (Figure 1) or with sample high viscosity (Figure 10b). Hence, the structural studies presented here do not confirm the presence of the gel−fluid transition region observed at low salt DMPG, but they suggest a more compact or packed structure in high salt DMPG promoted by barbaloin, as previously observed.[11]

In the presence of 20 mol % barbaloin, in accord with the absence of T_m^{off} in the low ionic strength DMPG DSC profile, and the dispersion high viscosity up to 50 °C, the low SAXS *q* value peak, typical of the DMPG anomalous temperature transition region, can be detected up to 60 °C (Figure 11b). Those experimental data strongly indicate that the presence of barbaloin prevents the low ionic strength DMPG dispersion to go to a "normal" fluid phase, up to 60 °C. It is interesting to point out that 20 mol % barbaloin reduces the fraction of the ESR feature characteristic of the more fluid and hydrated domain in the DMPG gel−fluid transition region (Figure 1, low salt at 25 °C), but does not eliminate it, as α-MSH does. Accordingly, the low SAXS *q* value peak, typical of the DMPG anomalous temperature transition region, is not present in α-MSH-containing samples (Fernandez et al., in preparation).

The effect of barbaloin of extending to higher temperatures the DMPG anomalous gel−fluid transition region is very interesting. For instance, if this anomalous region is related to the formation of bilayer pores,[7] or tattered bilayer fragments,[9] barbaloin could be preferentially interacting at the highly curved regions of DMPG bilayers (that would explain the decrease in the more mobile and polar 16-PCSL signal; Figure 1), in such a way as to stabilize those regions, preventing vesicle reorganization. However, the discussion of the structure of the DMPG anomalous phase is not the focus of the present work.

Conclusions

Here the effect of barbaloin on DMPG at high and low ionic strength was studied. DMPG at high salt, like most saturated lipids, presents a highly cooperative gel−fluid transition. Spin labels inside the bilayer indicate that barbaloin penetrates the membrane, slightly broadening the gel−fluid transition (Figures 2 and 5). Considering the 16-PCSL ESR spectra (Figure 1), viscosity, and SAXS measurements (Figures 10 and 11), it does not seem that the barbaloin−DMPG interaction makes the high ionic strength DMPG dispersion similar to the rather anomalous low ionic DMPG dispersion, as previously thought, despite the alterations caused on the DSC and turbidity thermal profiles.[11]

On the other hand, the effect of barbaloin on the gel phase of DMPG bilayers, at high and low ionic strength, is rather interesting and unusual. Its capacity to increase the packing of the hydrocarbon chains could be explained by its ability to bring the phospholipid head groups closer together. How could that be achieved? For instance, considering the OH groups present in the anthraquinone, one could speculate that barbaloin would be localized at the bilayer surface of DMPG gel phase, making hydrogen bonds with lipid head groups, forcing the lipids to get more packed.

At low ionic strength DMPG, the effect of barbaloin is quite surprising. Apart from increasing the gel phase packing, as mentioned above, the anthraquinone considerably increases the fluid lipid bilayer packing, also increasing the membrane polarity all over the hydrocarbon chain. Moreover, some of the characteristics of the anomalous DMPG gel−fluid transition are maintained and extended to temperatures up to 60 °C, like the sample high viscosity (Figure 10b) and the low *q* value interference SAXS peak (Figure 11b).

Hence, whereas barbaloin seems to be seated at the bilayer surface of DMPG gel phase, as discussed above, for higher temperatures, in the DMPG fluid phase, barbaloin is probably located close to the bilayer surface, due to its hydroxyl groups, but sufficiently inside the bilayer to restrain the hydrocarbon chain movement and to facilitate water penetration into the bilayer (Figures 3, 6, and 9). As suggested before,[11] on the basis of fluorescence-quenching measurements, the glucose moiety could be somewhere in the polar head region or in the interface, with the anthraquinone (chromophore) group in a deeper region responsible for the packing or restraining effect on the phospholipid acyl chains. As mentioned, it is interesting to keep in mind that DMPG can be regarded as a model system of anionic domains in natural membranes. Hence, some of the effects discussed here, concerning the barbaloin−lipid interaction, could be associated with the diverse activities of anthraquinone.

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