Interactions of the antimalarial amodiaquine with lipid model membranes

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ABSTRACT
A detailed molecular description of the mechanism of action of the antimalarial drug amodiaquine (AQ) is still an open issue. To gain further insights on that, we studied the interactions of AQ with lipid model membranes composed of dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylserine (DPPS) by spin labeling electron spin resonance (ESR) and differential scanning calorimetry (DSC). Both techniques indicate a coexistence of an ordered DPPS-rich domain with a disordered DPPC-rich domain in the binary DPPC/DPPS system. We found that AQ slightly lowered the melting transition temperatures associated to both domains and significantly increased the enthalpy change of the whole DPPC/DPPS phase transition. DSC and ESR data also suggest that AQ increases the number of DPPC molecules in the DPPC-rich domains. AQ also causes opposing ordering effects on different regions of the bilayer: while the drug increases the ordering of the lipid acyl chains from carbon 7 to 16, it decreases the order parameter of the lipid head group and of carbon 5. The gel phase was mostly affected by the presence of AQ, suggesting that AQ is able to influence more organized lipid domains. Moreover, the effects of AQ and cholesterol on lipid acyl chain ordering and mobility were compared at physiological temperature and, in a general way, they are similar. Our results suggest that the quinoline ring of AQ is located completely inside the lipid bilayers with its phenol ring and the tertiary amine directed towards the head group region. The nonspecific interaction between AQ and DPPC/DPPS bilayers is a combination of electrostatic and hydrophobic interactions.

1. Introduction
Malaria is still one of the most infectious diseases affecting the developing world. According to the World Health Organization (WHO), 655,000 people die every year due to malaria, out of which 86% are children under five years of age (WHO, 2011). One of the major barriers on fighting malaria is the worldwide drug resistance. Malaria parasites have developed resistance to all current drugs. To overcome drug resistance, WHO recommends the use of combined therapies with two or more drugs, in particular, the combination of amodiaquine (AQ) and artesunate (WHO, 2010). AQ is a 4-aminooquinoline antimalarial drug widely used in the past and whose chemical structure is similar to that of chloroquine (Olliaro et al., 1996; O’Neill et al., 1998). Several countries chose AQ as the first-line drug in combination with artesunate (WHO, 2010). In areas where artesunate resistance is low or absent, the therapeutic efficacy of artesunate–amodiaquine is highly correlated with the efficacy of AQ alone (Sá et al., 2009).

The mechanism of action of 4-aminooquinolines is not completely understood, but it is believed that is related to hemoglobin degradation in the digestive vacuole of the parasite (Pussard and Verdiere, 1994; O’Neill et al., 1998; Fitch, 2004). Hemoglobin is degraded by several enzymes of the parasite resulting in toxic heme metabolites. A process of detoxification converts heme into an insoluble crystal called hemozoin or “malaria pigment”, which participates in the parasite life cycle (Sherman, 1977). A complex formed by 4-aminooquinolines with heme (ferriprotoporphyrin IX) inhibits conversion of heme to hemozoin, thus killing the parasite. Although AQ forms complexes with ferriprotoporphyrin IX (Blauer et al., 1993), its complete mechanism of action, at a molecular level, remains unclear. In particular, there is not much information on how the drug overcomes the physical barrier represented by the cell membrane.

Membrane lipids are associated with vital cell functions. Besides being the structural blocks of cell membranes and the sources of metabolic energy, lipids play a central role in the regulation of innumerable cellular processes (Escribá, 2006;
Changes in the structure and lipid composition of membranes seem to be related to the development of several diseases (Lee, 2004). Although drug targets are usually proteins (Cohen, 2002; Overington et al., 2006), drugs can also bind to membrane lipids. Non-specific interactions of antiarrhythmics with myocardial membranes, for instance, are related to their mode of action (Gourley, 1971). Amlodipine, an antihypertensive drug, also interacts non-specifically with membranes (Mason et al., 1991). In a previous work, the non-specific interaction of the antimarial drug primaquine with biological membranes was suggested to represent an additional route in its overall mode of action and/or could be associated to its adverse effects (Basso et al., 2011). The importance of the non-specific drug-membrane interactions as a molecular event can be enhanced by a new therapeutic approach called membrane-lipid therapy. It is based on the binding of drugs to lipids, thus modulating the structure of membranes (Escribà, 2006). To understand the overall mechanism of action of AQ, studies of AQ-membrane interaction are important since the molecular targets of this drug, either proteins or lipids from membranes, are not completely known yet.

A combination of techniques is frequently used to study drug-membrane interactions (Seydel and Wiese, 2002). In particular, electron spin resonance (ESR) and differential scanning calorimetry (DSC), among several available techniques, have provided important contributions (Zhao et al., 2007; Budai et al., 2003; Könzöl et al., 2005; Pentak et al., 2008; Hamilton et al., 1991; Basso et al., 2011). Changes in membrane properties induced by drugs or other molecules are detected by lineshape alterations in the ESR spectra or by changes in heat exchange in DSC experiments (Duarte et al., 2008; Basso et al., 2011; Couto et al., 2011; Dyzsy et al., 2013).

In this paper, interactions of the antimarial drug AQ with lipid membrane models were investigated by ESR and DSC. The membrane models were constituted by an equimolar mixture of dipalmitylophosphatidylcholine (DPPC) and dipalmitylophosphatidylserine (DPPS), which are representative lipids of the parasite membrane (Hsiao et al., 1991). The effects of AQ on the melting behavior of DPPC/DPPS bilayers were monitored by DSC. Using spin labels attached to different positions along the lipid acyl chains and to the head group, we investigated the alterations induced by AQ on the ordering and dynamics of different regions of lipid bilayers by ESR. We also compared the effects caused by AQ with the effects caused by cholesterol on the structural dynamics of the binary system at physiological temperature. A possible drug location in the lipid bilayer was proposed.

2. Material and methods

2.1. Materials

The lipids 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phospho-1-serine (DPPS), cholesterol, and the spin labels 1-palmitoyl-2-stearoyl-(n-doxyl)-sn-glycero-3-phosphocholine (n-PCSL, where n = 5, 7, 10, 12, 14, and 16) and 1,2-dioleoyl-sn-glycero-3-phospho(temPO) choline (DOPTC) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). The antimarial drug AQ and the Hepes buffer (4-(2-hydroxyethyl)-1-piperizineethanesulfonic) were purchased from Sigma Chem., Co. (St. Louis). All reagents were used without further purification.

2.2. Methods

2.2.1. Lipid dispersion preparation

A lipid film was formed from a chloroform: methanol (2:1) solution of DPPC:DPPS (1:1) or DPPC:DPPS:cholesterol (10:10:1), and 0.5 mol% of spin label for ESR experiments, by drying under a stream of N2 and left under reduced pressure for 2 h to remove all traces of organic solvent. The film was then hydrated with 10 mM Hepes, at pH 7.4, to a lipid concentration of 20 mM, heated at 60 °C for 5 min, and vortexed. Part of the lipid dispersion was diluted with Hepes buffer to a final lipid concentration of 10 mM and another part was diluted with a 2 mM drug buffered solution to final lipid and drug concentrations of 10 and 1 mM, respectively. For ESR measurements with cholesterol-containing samples, a lipid film was formed from stock solutions of pure DPPC, DPPS, and cholesterol and the dried film was hydrated with Hepes to a final concentration of 10 mM of DPPC and DPPS and 1 mM of cholesterol.

2.2.2. Electron spin resonance

ESR measurements were performed with a JEOL FA-200 X-band spectrometer (JEOL Ltd., Tokyo, Japan). Temperature was controlled by a circulator bath that pumps fluid through an aluminum radiator attached to the ESR resonant cavity as described by Alouie and Smirnov (2006). The samples were placed into 1 mm inner diameter glass capillaries and then concentrated by centrifugation. The glass capillary was accommodated within a quartz tube. The acquisition conditions were: field modulation frequency, 100 kHz; field modulation amplitude, from 1 to 2 G; sweep width, 100 G; microwave power, 10 mW. Data presented here are an average of duplicate experiments and the uncertainties are the respective standard deviations.

2.2.3. Nonlinear least-squares (NLLS) simulations of ESR spectra

NLLS analyses of the ESR spectra were performed using the program Multicomponent (https://sites.google.com/site/altenbach/labview-programs/epr-programs/), a LabVIEW-based version (Altenbach, 2014) of the traditional scheme of Freed et al. for fitting ESR spectra of nitroxide spin probes (Schneider and Freed, 1989; Freed, 1976). The software calculates an ESR spectrum by solving the stochastic Liouville equation, allowing for the quantification of the structural dynamics of spin-labeled biomolecules in terms of motional rates and orientational ordering imposed by the local environment around the nitroxide radical.

The dynamics of spin-labeled lipids in lipid bilayers are better described by an axially symmetric rotational diffusion tensor, whose principal components, R1 and R2, represent the rotational diffusion rates around axes parallel and perpendicular, respectively, to the mean symmetry axis for the rotation, z0 (Schneider and Freed, 1989). For n-PC spin labels (n = 5, 7, 10, 12, 14, and 16), z0 is defined along the symmetry axis of the lipid, i.e., the fully extended direction of the acyl chains (see Fig. S1B of the Supporting information). For DOPTC, z0 is defined to be collinear to both the N–C4 and the N–O bonds of the tempo-choline group (Ge and Freed, 1998; Ge and Freed, 2003), which makes it also parallel to the magnetic z0 axis, in which the magnetic frame is defined (x0 axis points along the N–O bond, z0 lies along the 2p axis, y0 is perpendicular to both – see Fig. S1A). We found in our simulations that the spectra are not very sensitive to R1. Thus, to reduce the number of fitting parameters, N = R1/R2 was fixed to 1 and R = R2/R0 was varied instead. The mobility of the lipid acyl chains and the head groups was then reported as R0.

The calculated ESR spectra of the spin-labeled lipids are the result of the so-called MOMD (microscopic order-macroscopic disorder) model that takes into account the tendency of the spin labels to become partially ordered with respect to a local director z0 (defined as the normal vector to the bilayer surface), but these...
local directors are randomly oriented (macroscopically disordered) in the sample (Meirovitch et al., 1984). The microscopic molecular ordering of the spin labels is characterized by the order parameters $S_0$ and $S_2$, which are manifested through an orienting potential on the lipid head groups and the acyl chains that restricts the range of orientations of the spin probes (Schneider and Freed, 1989; Budil et al., 1996). $S_0$ is a measure of the inclination of $z_R$ with respect to the local director, $z_D$, whereas the nonsymmetric order parameter $S_2$ gives the deviation from cylindrical symmetry of the molecular alignment of $z_R$ relative to $z_D$. The larger the $S_0$, the smaller is the alignment, and the more restricted is the spin label motion.

The parameters involved in the simulations were: $g$-tensor ($g_{xx}$, $g_{yy}$, $g_{zz}$) and hyperfine-tensor ($A_{xx}$, $A_{yy}$, $A_{zz}$) components, rotational diffusion rates ($R_x$, $R_y$, $R_z$), orienting potential coefficients ($c_{20}$, $c_{22}$), and whenever needed, homogeneous Lorentzian or Gaussian linewidths. Seed values of the magnetic tensors were obtained from Earle et al. (1994), but small variations in their values were allowed during the fitting process. The strategy of the simulation follows that of Basso et al. (2011).

As for the uncertainties of the parameters from the NLLS analyses, we found an estimated error of $\pm 0.01$ for $S_0$ and $\pm 5\%$ for $R_z$ of n-PC spin labels, and of $\pm 0.006$ for $S_0$ and $\pm 3\%$ for $R_z$ of DOPTC. This means that we found good solutions for the stochastic Liouville equation with different sets of $c_{20}$, $c_{22}$ (only used in the simulations of DOPTC and 16-PCSL), and $R_z$. In general, good agreement between the experimental and theoretical spectra is obtained whenever $S_0$ and $R_z$ are both simultaneously increased within the uncertainties. Overall, the general trends observed in the ordering and dynamics of the spin labels for different sets of ($S_0$, $R_z$) values and for different lipid sample preparations were all consistent, even though the differences in $S_0$ and mostly in $R_z$ might be within the uncertainty of the corresponding parameters.

2.2.4. Differential scanning calorimetry

DSC traces were obtained by heating the samples from 5 to 70 °C at a scan rate of 20 °C/h with the Microcalorimeter VP-DSC (Microcal, Northampton, MA) or the calorimeter Nano-DSC-II (Calorimetry Sciences Corporation, Lindon, Utah, USA). Baseline subtractions and peak integrals were carried out using MicroCal Origin software.

3. Results and discussions

3.1. Calorimetric results show AQ-bilayer interaction

The effect of AQ on lipid membranes was investigated by determining the thermal behavior of a DPPC/DPPS mixture. Fig. 1 shows the DSC traces of DPPC/DPPS dispersions with and without AQ. Upon heating, the AQ-free samples exhibited a nonideal lipid mixing, as also reported by other authors (Sevcsik et al., 2008), characterized by a broad melting transition. Nonideal lipid mixing means that the lipids are not randomly distributed in the membrane but organized in clusters or domains. In this situation the bilayer displays lateral heterogeneity. This lateral phase separation was previously observed in DPPC/DPPS mixtures (Luna and McConnell, 1977; Gordon et al., 2006). DSC traces of AQ-free and AQ-containing samples presented two calorimetric peaks at temperatures $T_1$ and $T_2$ ($T_1 < T_2$, see the inset of Fig. 1). We suggest that these peaks could be attributed to the melting of lipids in two different domains, probably DPPC-rich and DPPS-rich domains. The thermograms also displayed very low enthalpic pre-transition peaks that are neither well defined nor reproducible and thus will not be discussed. The enthalpy change ($\Delta H$) of the whole transition and the values of $T_1$ and $T_2$ are presented in Table 1. As observed in Fig. 1, incorporation of AQ into the DPPC/DPPS lipid mixture increases the peak intensity at $T_1$, slightly lowers both $T_1$ and $T_2$, and significantly increases $\Delta H$ by $(20 \pm 2\%)$ on average (Table 1). In a previous work, a decrease of the transition temperature was also observed with primaquine, another aminoquinoline antimalarial drug, in DMPC bilayers (Basso et al., 2011). However, a much smaller amount of primaquine (lipid/drug molar ratio 91:1) was required to produce a similar effect than that caused by AQ (lipid/drug molar ratio 10:1). On the other hand, while the transition enthalpy of DMPC bilayers remained unchanged in the

![Fig. 1. DSC traces of samples with 10 mM DPPC/DPPS in Hepes buffer with (gray) and without (black) AQ. Inset: zoom of the calorimetric DSC peaks.](image)

**Table 1**

Calorimetric parameters of the phase transition of the binary DPPC/DPPS membranes obtained from the DSC traces.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$T_1$ (°C)</th>
<th>$T_2$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC/DPPS</td>
<td>17.4</td>
<td>43.23</td>
<td>44.39</td>
</tr>
<tr>
<td></td>
<td>18.9</td>
<td>43.19</td>
<td>44.40</td>
</tr>
<tr>
<td>DPPC/DPPS with AQ</td>
<td>21.3</td>
<td>43.00</td>
<td>44.20</td>
</tr>
<tr>
<td></td>
<td>22.2</td>
<td>43.00</td>
<td>44.12</td>
</tr>
</tbody>
</table>
Fig. 2. Experimental (black) and best-fit (gray) ESR spectra of the head group spin label DOPTC and the acyl chain n-PCSL (n = 5, 7, 10, 12, 14, and 16) in DPPC/DPPS membranes in the absence (left) and in the presence (right) of AQ. (a) One-component nonlinear least squares fits of DOPTC, 5-, 7-, 10-, and 12-PCSL ESR spectra. (b) Spectra of 14- and 16-PC spin labels were simulated with two components. The arrows show the second, more mobile, and less populated component. The spectra were acquired at 36 °C.
presence of primaquine, AQ induced a large increase in \( \Delta H \) of DPPC/DPPS membranes, suggesting different drug-lipid interactions and thus different effects on membranes.

Our DSC results show a clear interaction between AQ and the lipid membranes, since AQ changes the shape of the thermograms, decreases the melting temperatures and increases the transition enthalpy of the binary system. These results could indicate a penetration of AQ into the DPPC/DPPS bilayers, since molecules that penetrate lipid bilayers usually lower the gel-to-fluid phase transition (Jain and Wu, 1977). As mentioned before, the narrow DSC peak at \( T_1 \) (Fig. 1) can be due to the melting of either DPPC-rich or DPPS-rich domains. Using infrared spectroscopy, Schultz et al. showed the coexistence of DPPC-rich and DPPS-rich aggregates in a binary DPPC/DPPS membranes and also showed that DPPC molecules melt at a temperature (45.2 \( ^\circ \)C) lower than that of DPPS (47.8 \( ^\circ \)C) in this lipid system (Schultz et al., 2009). Thus, we propose that the peak at \( T_1 \) in our DSC thermograms might be due to the melting of DPPC in DPPC-rich domains, whereas the peak at \( T_2 \) could be related to the melting of DPPS in DPPS-rich domains. Furthermore, lipids do not melt independently of each other but rather simultaneously in clusters of a certain amount of lipids (Heimburg, 2007). Thus, the melting of lipids is a cooperative phenomenon and the cooperativity is proportional to the number of lipids in a cluster or domain. The larger the cooperativity, the narrower and more intense the transition peak. The intensity increase of the DSC peak at \( T_1 \) caused by AQ binding to the binary system is therefore likely due to an increase of the number of lipids in the domains melting at \( T_1 \), that is, the size of the DPPC-rich domains increases. Since there is a direct correlation between membrane curvature and both enthalpy changes (Yokoyama et al. (2013)) and lateral phase separation (Duwe et al., 1990; Sackmann, 2006), the aforementioned effects of AQ on the lipid mixture could be explained by an increase in DPPC/DPPS membrane curvature.

### 3.2. ESR results suggest AQ penetration into the bilayer

Changes in the order and dynamics of the membrane caused by AQ were investigated by means of ESR spin labeling technique at physiological temperature. The hydrophobic region of the bilayer was monitored by using spin labels located at different depths along the lipid acyl chain. Fig. 2a and b shows the experimental and simulated ESR spectra of the head group spin label DOPTC and the acyl chain spin labels n-PCSL (n = 5, 7, 10, 12, 14, and 16) acquired at 36 \( ^\circ \)C in the absence and in the presence of AQ. Generally, the experimental and best-fit theoretical spectra were in very good agreement and considerable changes were observed in all ESR spectra upon incorporation of AQ. Except for the 14- and the 16-PCSL signals (Fig. 2b), all other ESR spectra were simulated with only one component (one spin label population). The two-component feature in the 14- and 16-PCSL spectra will be discussed below.

Fig. 3 presents the order parameter (\( S_0 \)) profile obtained from the NLLS simulations of the ESR spectra displayed in Fig. 2. Overall, NLLS fits show that AQ increases the ordering and slightly decreases the rotational mobility of the whole hydrophobic region of the lipid bilayers, except for the DOPTC and for 5-PCSL, in which an opposite effect was observed: for example, AQ slightly increases \( S_0 \) (from 0.711 to 0.692 – Table S2) and significantly increases \( R \) (from 3.31 to 4.36 \( \times 10^5 \) \( \text{s}^{-1} \)) for 5-PCSL. Interesting features were reported by some of the spin labeled lipids and will be discussed in more details.

NLLS simulations of the ESR spectra of the head group spin label DOPTC provided information on the cooperativity, the orientation of the nitroxide radical at the bilayer surface. With a positive value for \( S_0 = 0.518 \) and a negative value for the nonaxial order parameter \( S_2 = -0.084 \), the orientation of the trimethyl ammonium (TMA) group of the DOPTC in the binary DPPC/DPPS system is almost similar to that of the dipalmitoylphosphatidyl temp (2,2,6,6-tetramethyl-1-oxo) choline (DPPTC) in a pure DPPC dispersion in the gel phase (\( S_0 = 0.50 \) and \( S_2 = -0.22 \) at 25 \( \text{C} \)) (Ge and Freed, 1998) or to that of 4-O-(1,2-dipalmitoyl-sn-glycero-3-phospho)-4-hydroxy-2,2,6,6-tetramethyl-piperidin-1-oxo (DPP-Tempo) in dioleoylphosphatidylcholine (DOPC) dispersions at \(-11 ^\circ \text{C (} S_0 = 0.516 \) and \( S_2 = -0.238 \)) in the presence of excess water (Ge and Freed, 2003). This means that the principal diffusion axis (\( z_0 \)), which coincides with \( x_0 \) (parallel to the N–O bond – see Section 2.2.3), is preferentially aligned along the local director of the bilayer surface. The main difference in the orientation of the TMA group of DOPTC in DPPC/DPPS membranes compared to that of the other spin labels (DPPTC in DPPC or DPP-Tempo in DOPC) is the lower preference of either \( x_0 \) or \( y_0 \) axes to be oriented perpendicular to director, due to the low value of \( S_2 \) (\( -0.084 \)). To make this clear, we decomposed the spherical tensor components \( S_0 \) and \( S_2 \) in their Cartesian counterparts (Ge and Freed, 2003): \( S_{2x} = S_0 = 0.518 \), \( S_{2y} = 1/2\sqrt{3}S_{2z} - S_0 = -0.310 \), and \( S_{2z} = -1/2\sqrt{3}/2S_{2x} + S_0 = -0.207 \). As discussed before, the high positive value of \( S_{2x} \) means a strong preference for \( z_0 \) to be aligned normal to the bilayer, whereas negative and close values of \( S_{2x} \) and \( S_{2y} \) indicate an almost equal preference for \( x_0 \) and \( y_0 \) diffusion axes to be oriented tangential to the bilayer. The addition of AQ to DPPC/DPPS samples changes the ordering and dynamics of DOPTC, which means that AQ binds to and interacts with the lipid head group of the bilayers. Interestingly, AQ significantly decreases both the perpendicular rotational mobility \( R \) (from 3.02 to 2.45 \( \times 10^5 \) \( \text{s}^{-1} \)) and the order parameter \( S_0 \) (from 0.518 to 0.452) of the spin label. The latter result implies that lipid-AQ interaction increases the average angular amplitude of the wobbling motion of the nitroxide moiety (the larger \( S_0 \), the smaller the amplitude). In terms of the Cartesian components, \( S_{2x} = -0.291 \), \( S_{2y} = -0.160 \), and \( S_{2z} = -0.452 \), we can also infer that AQ binding to membranes induces \( z_0 \) to be much less oriented along the local director, whereas \( x_0 \) and \( y_0 \) axes retain their orientation tangential to the bilayer normal, but \( y_0 \) becomes less oriented.

Another interesting feature observed in Fig. 2a is that the spectrum of 12-PCSL from the AQ-free sample presented a much broader line than would be normally expected for this spin label. This clearly indicates strong spin–spin interactions that likely arise from cluster formation of 12-PCSL molecules in the DPPC/DPPS membranes. Partial segregation of spin labels was also noted, for instance, by Fajer et al. for n-PCSL in the DPPC gel phase in a
saturation transfer ESR study (Fajer et al., 1992) and by Earle et al. in a rigid-limit 250 GHz ESR study of n-PCSL incorporated in DPPC and in palmitoyl-oleoyl-PC (POPC) lipid vesicles (Earle et al., 1994). In fact, the best least-square fits to 12-PCSL ESR spectra were achieved by allowing for a Heisenberg exchange coupling in the simulations, which yielded a $a_{0\text{HH}} = 4.9 \times 10^{-7}$ s$^{-1}$. Interestingly, addition of AQ into the preformed 12-PCSL-clustered DPPC/DPPS lipid vesicles abolished the spectral broadening, thus suggesting disruption of those clusters. Direct lipid-AQ interactions might promote a better miscibility of the 12-PCSL molecules in the binary system.

A distinct behavior was observed in the spectra of 14- and 16-PCSL (Fig. 2b), which exhibited additional spectral features (see the low-field and high-field lines) characteristic of a second spin population, indicating the coexistence of two types of domains in the 1:1 DPPC/DPPS lipid bilayers, which is in agreement with our DSC results. A two-component system was also observed, for instance, by Basso et al. in the rippled phase of dimyristoyl-PC (DMPC) lipid bilayers (Basso et al., 2011) and by Chiang et al. in a ternary DPPC/dilauroyl-PC/cholesterol (DPPC/DLPC/Chol) lipid mixture (Chiang et al., 2004). Our result shows that ESR is very sensitive to detect the heterogeneity of different micro-environments in a binary system. Thus, an extra ESR signal with different ordering and rotational dynamics was added to the fitting process. From the NLLS fits, we found that the most populated component of the two spin labels presented the higher order parameter: $S_0$ of 0.367 for 14-PCSL (87% population) and of 0.343 for 16-PCSL (93% population). $S_0$ values of the less ordered domain were 0.196 for 14-PCSL (13% population) and 0.117 for 16-PCSL (7% population).

As shown in Fig. S2 of the Supporting information, the theoretical 16-PCSL spectrum of the ordered domain (component 1) in the binary system is very similar to the experimental spectrum of this same spin label in pure DPPS vesicles at the same temperature. Similarly, the theoretical spectrum of the less ordered component resembles, despite the difference in intensity, that of the 16-PCSL incorporated in pure DPPC lipid bilayers at the same temperature (Fig. S2). Thus, the coexistence of the ordered and disordered regions in the binary DPPC/DPPS lipid system is most likely due to the coexistence of DPPS-rich (ordered) and DPPC-rich (disordered) domains.

The slight differences in the spin population values of the ordered and disordered domains for 14- and 16-PCSL suggest that these spin-labeled lipids have different partition coefficients $K_P$ between those domains. Since the mole fraction of 14-PCSL in the less ordered state is higher than the population of 16-PCSL in this same domain (0.13 and 0.07, respectively), 14-PCSL must partition better in the disordered domains than 16-PCSL. In fact, a slighter preference of 14-PCSL for the disordered phase compared to 16-PCSL in a two-phase coexisting region of a ternary system has been reported (Swamy et al., 2006).

Upon AQ addition to the lipid samples, changes in the lineshape of the 14- and 16-PCSL are observed (Fig. 2b). The nonlinear least-squares fits show that binding of AQ to the DPPC/DPPS membranes increases the ordering ($S_0$: from 0.367 to 0.413 for 14-PCSL and from 0.343 to 0.379 for 16-PCSL) and decreases the perpendicular rotational diffusion rate ($R_\parallel$: from 6.76 to 6.31 $\times 10^{-10}$ s$^{-1}$ for 14-PCSL and from 2.09 to 1.78 $\times 10^{-10}$ s$^{-1}$ for 16-PCSL) of the DPPS-rich domains (Table S2). The overall result is an increased packing of the bilayer center of the DPPS-rich domain, whereas almost no change was observed on ordering and dynamics of the center of the hydrophobic core of the DPPC-rich domains (Table S2). However, both 14- and 16-PCSL spectra of the AQ-containing samples show an increase of the spin-labeled lipid population of the less ordered DPPC-rich domain (from 0.13 to 0.28, for 14-PCSL, and from 0.07 to 0.17, for 16-PCSL). The redistribution of the spin label molecules between the two domains upon AQ addition might be a result of two main effects: (1) changes in the equilibrium constant between the DPPC-rich and DPPS-rich domains and/or (2) changes in the partition coefficient of the spin labels between the two regions. We explored these two possibilities as follows. Firstly, we defined the partition coefficient $K_P$ as the ratio of spin-label concentration in the ordered ($L_o$) and disordered ($L_d$) domains as in Swamy et al. (2006):

$$K_P = \frac{P(L_o)}{P(L_d)}$$

where $P(L_o)$ and $P(L_d)$ are the populations of the spin labels incorporated in the disordered ($L_d$) and ordered ($L_o$) domains, respectively, obtained from the NLLS fits to the ESR spectra, and the second term on the right hand side of the equation represents the percentage of lipids in the DPPC-rich ($L_o$) and DPPS-rich ($L_d$) domains. $K_P$ would be unity if spin labels partition equally into each domain. If we further suppose that the relative populations (percentage of lipids) of the ordered and disordered domains are characteristic of the binary system and thus do not change with the addition of 0.5 mol% of the spin label (this is reasonable since the acyl chain of spin-labeled lipids are almost identical to that of DPPC and DPPS), the ratio $K_P$ values for 14- and 16-PCSL can be directly calculated as the ratio of the spin label populations obtained from the NLLS fits:

$$K_P = \frac{S_0^{14PC}}{S_0^{16PC}}$$

Thus, $K_P$ gives a good estimate of the relative partition coefficient of both spin labels into the $L_o$ and $L_d$ phases. We found that $K_P = 0.504$ for the AQ-free sample and $K_P = 0.527$ for the AQ-containing sample, that is, the difference is within the uncertainty of the spin label populations obtained from NLLS simulations, which is $\pm 3\%$. This result suggests that the partitioning of the spin labels in the ordered and disordered domains does not depend on the AQ binding to membranes at 36 °C (at least on the specific conditions of our experiments). Consequently, changes on spin label population upon AQ binding reflect changes on the equilibrium constant between DPPC-rich and DPPS-rich domains in the binary system. As mentioned before, our DSC experiments suggest an increase of the cooperativity of the DPPC-rich domains upon AQ incorporation (see Section 3.1). Our ESR results also support this hypothesis. Binding of AQ to DPPC/DPPS membranes could shift the equilibrium constant between DPPC-rich and DPPS-rich domains towards the DPPC-rich one. As a result, there may be an increase in the number of lipids in the DPPC-rich clusters, which can contribute to the increased cooperativity of the phase transition of this domain.

Taken together, the ESR/NLLS results suggest that AQ inserts into the DPPC/DPPS membranes and changes the molecular force field, i.e., the orienting potential, in the headgroup and in the acyl chain regions. We suggest that both the phenol ring and the protonated tertiary amine of AQ (at pH 7.4, AQ is mostly monoprotonated) are most likely located in the headgroup region of the lipid bilayer. This can enhance the hydrogen bond network of that region (due to additional hydrogen bonds between AQ and the lipid head groups) and also decrease electrostatic repulsion among PS in DPPS-rich domains. Direct AQ-head group interaction via the aforementioned groups also changes the orientation of the TMA group of the DOPTC spin label, which impacts the membrane orienting potential. Additionally, except for the local environment around carbon 5, the increased $S_0$ values of almost the entire acyl chain region indicate an increased lateral packing density of the lipids induced by AQ, which can enhance van der Waals interactions between lipids. This increased bilayer
ordering is consistent with a higher transition enthalpy, since more heat is required to break molecular interactions and melt the lipids. The slight reduction of $S_0$ with a remarkably increase of rotational mobility around carbon 5 – as shown by NLLS simulations of 5-PCSL spectra – can be explained by the insertion of the quinoline ring of AQ into the lipid matrix. With the quinoline ring located into the hydrophobic core of the lipid bilayer, but close to the polar–apolar interface, and the phenol ring OH and the tertiary amine directed towards the head group, a free volume could be created around the very first carbon atoms of the lipid acyl chains, which can explain the markedly increase of the rotational degree of freedom of 5-PCSL. Insertion of the quinoline structure into lipid matrix may also enhance van der Waals attractions between lipids, which can contribute to increase the lateral packing. In fact, insertion of both unprotonated and protonated forms of the quinoline ring of another 4-aminoquinoline antimalarial drug, chloroquine, into the apolar region of an anionic micelle has been also proposed (Santos et al., 2014; Usman and Siddiq, 2013).

To gain further insights on the hypothesis of AQ penetration into the membrane, as strongly suggested by our results, we also compared the effect of AQ on acyl chain order and mobility with those provoked by cholesterol. It is well known that cholesterol is located within the lipid bilayers (Huang, 1977; Ahmad and Mellors, 1978; Subczynski et al., 1992) and, as will be discussed further, cholesterol also causes no effect on the ordering of the head groups ($S_0 = 0.52$; $S_2 = -0.11$), but slightly reduces the rotational mobility (from 3.16 to 3.02 $\text{s}^{-1}$) as monitored by DOPTC spin label. Despite the small difference in $R_\theta$ (4.4%), this variation is higher than the uncertainty calculated for this parameter (±3% – see Section 2.2.3). Also, the observed trend of reduced head group mobility in cholesterol-containing samples is obtained in different lipid preparations: there is a consistent decrease of the ratio between the intensity of the low-field and the center-field lines of the DOPTC ESR spectrum in the presence of cholesterol.

Changes in lipid order parameters can be used to infer how deep foreign molecules go inside the lipid bilayers (Biaggi et al., 1997). To gain further insights into the localization of AQ inside DPPC/DPPS bilayers, we plotted the variation of $S_0$ induced by AQ or cholesterol

![Fig. 4. Experimental (black) and best-fit (gray) of ESR spectra of the head group spin label DOPTC and the acyl chain n-PCSL ($n = 5, 7, 10, 12, 14, \text{and } 16$) in either DPPC/DPPS (left) or DPPC/DPPS/Chol (right) membranes. Spectra of 14- and 16-PC spin labels were simulated with two components. All the spectra were acquired at 36 °C.](image-url)
system (Fig 6a). Above the phase transition, there is no change in the lineshape of the spectra due to addition of AQ. However, cholesterol slightly decreases the ratio between the high-field and the center-field lines, as indicated by the arrow in the figure, which means that 5 mol% of Chol slightly decreases the mobility and/or increases the ordering of the region monitored by 14-PCSL. On the other hand, below the phase transition, both AQ and cholesterol broaden the spectrum relative to the pure lipid mixture. This means that both molecules increase the bilayer ordering, but the effect caused by AQ is stronger than that of cholesterol, which corroborates our initial hypothesis. The lineshape alterations in the 14-PCSL spectrum caused by incorporation of AQ into the inner and outer monolayers (Fig 6a) are even more pronounced than those caused by incorporation of the drug only into the outer monolayer (see Fig. 2b). The effect of cholesterol on the heat capacity of a DPPC/DPPS/Chol lipid dispersion was also similar to the effect caused by AQ, though there was no change in the transition enthalpy (Fig. 6b). Despite the low miscibility of cholesterol in phosphadityl serines (PS) bilayers reported by several authors (Bach and Wachtel, 2003), the effect of limiting amounts of cholesterol on the thermotropic behavior of binary phospholipid mixture indicated that anionic phospholipids exhibit greater affinity for cholesterol than do the zwitterionic phospholipids PC and PE (Van Dijck, 1979). McMullen et al. (2000) also showed that cholesterol exhibits greater miscibility in anionic PC than in uncharged glycolipid bilayers. A personal communication published in the McMullen et al. paper (McMullen et al., 2000) reports an up to 67 mol% cholesterol solubility in 1-palmitoyl-oleoyl-PS bilayers. Cholesterol incorporation, up to 15 mol%, in the positively charged DODAB bilayers was also reported (Benatti et al., 2007). We used a cholesterol concentration of 5 mol% of the total lipid and, considering its reported higher affinity to PS compared to PC, we suggest that cholesterol partitions better in PS-rich domains than in PC-rich ones.

The incorporation of cholesterol in the bilayer is related to hydrophobic effects between the cholesterol and the lipid bilayer. Since AQ is a hydrophobic drug, and it is worth noting that its hydrophobicity has been linked to its antimalarial activity (Elslager et al., 1964), we expect that hydrophobic effects between AQ and the bilayers could also take place. However, besides the hydrophobic effect, electrostatic forces between AQ and the negatively charged DPPC/DPPS bilayers may occur, since at the pH studied, about 98% of AQ was in the monoprotonated form (Naisbitt et al., 1997). To verify the role of the bilayer charge in the interaction with AQ, we measured the effect of the same amount of AQ on the ESR spectrum of 12-PCSL in DPPC/DPPS bilayers with

![Fig. 5.](image_url) Variation of the order parameter, $S_n$, relative to the pure lipid bilayer as a function of nitroxide position, $n$, of n-PCSL in DPPC/DPPS membranes in the presence of AQ or cholesterol. Temperature: 36 °C.

![Fig. 6.](image_url) (a) ESR spectra of 14-PCSL in DPPC/DPPS membranes in the absence and in the presence of AQ or Chol, in the gel phase (left) and fluid phase (right). (b) DSC traces of 10 mM DPPC/DPPS in Hepes buffer with (gray) and without (black) cholesterol.
me are amphiphilic, such as AQ, chloroquine, primaquine, quinacrine, important role (Schreier et al., 2000). Since several antimalarials specifically active compounds confers them different types of mechanism of action. 3.3. Possible role of drug-membrane interactions to the antimalarial mechanism of action

The amphiphilic or hydrophobic character of many pharmacologically active compounds confers them different types of association, such as self-association and specific and/or non-specific binding to proteins and lipid membranes. Usually, the plasma membrane is one of their sites of action and, even if the target is intracellular, the interaction with this first barrier plays an important role (Schreier et al., 2000). Since several antimalarials are amphiphilic, such as AQ, chloroquine, primaquine, quinacrine, mefloquine, and quinine, drug-membrane interactions may contribute to their whole mechanism of action. Indeed, interactions between halofantrine and lipid bilayers of PC and PE were observed by DSC measurements (Lim and Go, 1999). Halofantrine caused a broadening of the phase transition of PC bilayers and a decrease in both enthalpy and transition temperature. A similar effect of halofantrine was observed with PE. Our results also showed a decrease in the transition temperature caused by AQ. In another study, the effects caused by halofantrine on the phase transition of DPPC bilayers were compared with those caused by quinine and mefloquine (Lim and Go, 1995). Similar to halofantrine, the latter drugs also interacted with DPPC bilayers, changing the phase transition and decreasing the transition temperature. Pajeva et al. showed that quinacrine was also able to interact with membranes, since the drug caused changes in the thermotropic behavior of DPPS bilayers by decreasing the transition enthalpy (Pajeva et al., 1996). Primaquine-membrane interactions were shown by a combination of pressure perturbation calorimetry, DSC and ESR (Basso et al., 2011). It was shown that primaquine stabilizes the fluid phase of different lipid model membranes, causing a local disorder on the acyl chains of the lipids. This fluidizing effect seems to be due to the interaction of primaquine with the lipid head groups, causing a less dense gel phase. Interactions of mefloquine, quinacrine, chloroquine, and quinine with DPPC bilayers were investigated by $^3$H and $^{31}$P NMR (Zidovetzki et al., 1989). Although chloroquine did not seem to affect the bilayer structure of DPPC, a weak chloroquine–DPPC interaction could not be disregarded by the authors. Their results also pointed that quinacrine interacts only with the surface of the DPPC bilayers, whereas both quinine and mefloquine penetrate into the membrane. A further investigation of DPPC–chloroquine interactions using a fluorescent probe inserted into the bilayer showed that chloroquine rigidified DPPC membranes (Ghosh et al., 1995). Our results also show a rigidifying effect on the hydrophobic core of the bilayers caused by AQ.

It is believed that the heme is the target of both AQ and chloroquine, since these drugs prevent heme detoxification. The parasite protects itself against the toxicity of AQ by polymerizing it to insoluble hemozoin. As weak bases, AQ and chloroquine accumulate in the acidic environment of the parasite’s food vacuole and inhibit heme polymerization. After exiting the food vacuole, the heme that is not polymerized is degraded by glutathione. Ginsburg et al. found that AQ and chloroquine competitively inhibit the degradation of heme by glutathione, allowing it to accumulate into the membranes (Ginsburg et al., 1998). Heme, which is released as ferriprotoporphyrin IX (FPPIX) in the presence of oxygen (Jacob and Winterhalter, 1970), disrupts the membrane barrier properties and disturbs the ion homeostasis in the cells, which kills the parasites. Famin et al. showed that the inhibition constant of glutathione-mediated destruction of heme in ghost membranes by AQ is 25% greater than by chloroquine (Famin et al., 1999). It was supposed that this difference might be related to different locations of AQ, chloroquine, and FPPX within the membranes. It was proposed that chloroquine is located mainly in the head group region of the lipid bilayer, but not intercalated into the phospholipids (Ginsburg and Demel, 1983; Kirsch et al., 1983 Zidovetzki et al., 1989). However, AQ is much more hydrophobic than chloroquine (the partition coefficient of AQ in octanol is 359% greater than that of chloroquine, according to Bray et al., 1996) and thus it may intercalate in the bilayer, as we showed in the present paper. In this way, the non-specific interaction between lipids and AQ, as other antimalarial drugs, may have an important role in their overall and complex mechanism of action.

4. Conclusions

We showed that AQ is able to interact non-specifically with DPPC/DPPS lipid bilayers affecting the gel phase more than the lipid fluid phase. AQ seems to influence more organized lipid domains, since the fluid phase of the model membrane investigated was not affected. This AQ-bilayer interaction is driven by a combination of electrostatic forces and hydrophobic effects. Firstly, the approximation between the drug and the bilayer surface is driven by electrostatic forces between the positively charged amino group of AQ and the negatively charged PS headgroups. Once close the bilayer, hydrophobic effects also take place. Our results indicate that AQ binding to the binary DPPC/DPPS system shifts the equilibrium constant between the disordered DPPC-rich and the ordered DPPS-rich domains.
towards the DPPC-rich one. We suggest that AQ is located completely inside the bilayer with the positively charged amino group and the OH of the phenol group pointing outwards to the polar groups of the bilayer.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemphyslip.2014.12.003.

References


