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Journal

Photochemistry and Photobiology, 84(1)

ISSN

0031-8655

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Publication Date

2008

DOI

10.1111/j.1751-1097.2007.00221.x

Photochemistry and Photobiology, 2008, 84: 209–214

Fluorescence Studies of the Interactions of Ubiquinol-10 with Liposomes

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Received 25 July 2007, accepted 12 September 2007, DOI: 10.1111/j.1751-1097.2007.00221.x

ABSTRACT

Ubiquinone-10 plays a central role in energy production and its reduced form, ubiquinol-10 is also capable of acting as a potent radical scavenging antioxidant against membrane lipid peroxidation. Efficiency of this protection depends mostly on its localization in lipid bilayer. The intrinsic fluorescence of ubiquinol-10 and of the exogenous probe, Laurdan, has been used to determine the location of ubiquinol-10 in unilamellar liposomes of egg phosphatidylcholine (EggPC) and dimyristoyl phosphatidylcholine. Laurdan fluorescence moiety is positioned at the hydrophilic–hydrophobic interface of the phospholipid bilayer and its parameters reflect the membrane polarity and microheterogeneity, which we have used to explore the coexistence of microdomains with distinct physical properties. In liquid–crystalline bilayers ubiquinol has a short fluorescence lifetime (0.4 ns) and a high steady-state anisotropy. In a concentration-dependent manner, ubiquinol-10 influences the Laurdan excitation, emission and generalized polarization measurements. In EggPC liposomes ubiquinol-10 induces a decrease in membrane water mobility near the probe, while in dimyristoyl liposomes a decrease in the membrane water content was found. Moreover the presence of ubiquinol results in the formation of coexisting phospholipid domains of gel and liquid–crystalline phases. The results indicate that ubiquinol-10 molecules are mainly located at the polar-lipid interface, inducing changes in the physico-chemical properties of the bilayer microenvironment.

INTRODUCTION

Ubiquinone-10 (UQ) is an integral redox and proton-translocating component of the mitochondrial electron transport chain (1) and it is also widely distributed in other subcellular membranes (2).

It has been well established that ubiquinol-10 (UQH_2), a reduced form of UQ (Scheme 1), protects either membrane phospholipids and serum low-density lipoproteins from lipid peroxidation or mitochondrial membrane proteins and DNA from free-radical-induced oxidative damage (3). The membrane presence of enzymes capable of regenerating ubiquinol through the reduction of ubiquinone, or any ubisemiquinone radicals, is critical for effective UQH_2 antioxidant activity (4). UQH_2 can act both directly, by preventing the formation of lipid peroxyl radicals and indirectly by regenerating α-tocopherol (5).

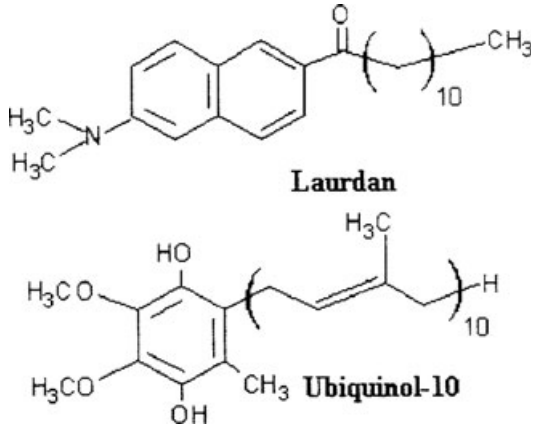
UQH_2-derived semiquinones can also generate superoxide radicals, initiating a variety of prooxidative reactions and as this depends on the membrane localizations of UQH_2 (6), its net antioxidative capacity is strongly dependent on its membrane position.

Despite many studies by different techniques such as fluorescence anisotropy and quenching of fluorescent probes (7), infrared spectroscopy (8) and differential scanning calorimetry (9), its location inside the membrane is still a matter of discussion. Recently, the intrinsic fluorescence of reduced plastoquinols and α-tocopherol have been used to observe their incorporation in liposomes (10).

In this work, intrinsic fluorescence of UQH_2 as well as the fluorescence of Laurdan (Scheme 1), an exogenous probe, incorporated in egg phosphatidylcholine (EggPC) and dimyristoyl unilamellar liposomes have been used to elucidate UQH_2 location in the bilayer.

MATERIALS AND METHODS

Phospholipid vesicle preparation. Egg phosphatidylcholine and dimyristoyl phosphatidylcholine (DMPC) were from Avanti Polar Lipids (Alabaster, AL). Small unilamellar vesicles containing UQH_2 were prepared as previously described (7). Large unilamellar vesicles (LUVs) were prepared by extrusion through 0.1 μm polycarbonate filters, using LiposoFast apparatus (Avestin, Inc., Canada) (11).



Scheme 1. The chemical structure of ubiquinol-10 and Laurdan.

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UQH_2 was obtained reducing ubiquinone (a kind gift from Kaneka, Osaka, Japan) in ethanol with sodium borohydride. The degree of UQH_2 incorporation was evaluated after one pentane washing of the samples according to Degli Esposti *et al*. (12).

Fluorescence measurements. Ubiquinol steady-state fluorescence anisotropy measurements were performed by using an ISS Photon Counting Spectrofluorometer, model PC1 (Champaign, IL). The excitation and emission wavelength were 290 and 360, respectively, and the excitation and emission bandwidths were 2 nm. A Schott 320 cutoff filter was used in the emission path. The computer program calculated fluorescence anisotropy by using the expression $[I_V-(I_{hu} \times g)]/[I_V + (2I_{hu} \times g)]$ where g is an instrumental correction factor, I_V and I_{hu} are respectively the emission intensities with the polarizers parallel and perpendicular to the direction of the polarized exciting light. The anisotropy values represent the averaged values from three different samples.

UQH_2 fluorescence lifetime measurements were performed at 25°C on an ISS K2 multifrequency phase and modulation fluorometer (Champaign, IL). The samples were excited using a 293 nm beam from a frequency-doubled, Nd:YAG (Coherent Antares)-pumped, rhodamine dye laser (Coherent). A Schott WG 320 long-pass filter was placed in the emission path to eliminate scatter of the excitation beam and to collect fluorescence above 310 nm. Measurements were performed using 14 modulation frequencies ranging from 10 to 300 MHz. All lifetime measurements were obtained using *2,2′*-p-phenylene-bis-(5-phenyl)oxazolone (POPOP) in the reference cell. The POPOP lifetime was 1.35 ns. Phase and modulation data were fitted with a double exponential decay model using a nonlinear least-squares procedure. The program minimized the reduced chi-square defined by an equation reported elsewhere (13).

Laurdan steady-state fluorescence measurements were performed on a Perkin-Elmer LS55 spectrofluorimeter. Phospholipids and Laurdan solutions were mixed to a final probe-lipid molar ratio of 1:1000. UQH_2 in ethanol was added to LUVs at different molar ratios. As UQH_2 was always dissolved in 5 μL of ethanol, control experiments were performed with the same volume of this solvent. Fluorescence from liposome sample without Laurdan was always subtracted from the data. The fluorescent probe is located at the hydrophobic–hydrophilic interface of the bilayer (at the glycerol backbone) (14) and its spectral features are largely sensitive to the polarity and molecular dynamics of solvent dipoles in its microenvironment (15,16). The polarity and dynamics of the dipoles surrounding the fluorescent moiety of Laurdan are very different in the gel and in the liquid–crystalline phases of phospholipids (15,16). Below the main phase transition temperature (T_m; *i.e*. in the gel phase), the Laurdan emission maximum is near 440 nm, whereas above the T_m (*i.e*. in the liquid–crystalline phase), this maximum is shifted to 490 nm (16). Laurdan emission and excitation generalized polarization (GP) spectra were derived by calculating the GP value for each emission and excitation wavelength as follows (17):

$$\text{Em GP} = (I_{410} - I_{340})/(I_{410} + I_{340})$$

where I_{410} and I_{340} are the intensities at each emission wavelength, from 420 to 550 nm, obtained using a fixed excitation of 410 and 340 nm, respectively.

$$\text{Ex GP} = (I_{440} - I_{490})/(I_{440} + I_{490})$$

where I_{440} and I_{490} are the intensities at each excitation wavelength, from 320 to 420 nm, obtained using a fixed emission wavelength of 440 and 490 nm, respectively (17). The choice of 410, 340, 440 and 490 nm for GP calculation was based on the characteristic excitation and emission wavelengths of pure gel and liquid–crystalline lipid phases (18). All spectra were normalized.

RESULTS

Intrinsic UQH_2 fluorescence

Normalized fluorescence emission spectra of different concentrations of UQH_2 (1, 2, 3.2, 10, 20 mol.%) in EggPC small unilamellar vesicles are reported in Fig. 1. The emission maxima, F_{max}, show a slight redshift on increase in the UQH_2 content ranging from 358 nm for the lowest concentration to 370 nm for the highest. At all concentrations used for the experiments we did not notice any decrease in UQH_2 fluorescence intensity, indicating no aggregation of the molecules. The same samples were used to study UQH_2 steady-state fluorescence anisotropy (r_s) in EggPC and DMPC small unilamellar liposomes at 30°C (Fig. 2). At this temperature both the phospholipids are in the fluid state. In DMPC liposomes UQH_2 r_s shows higher values than those in EggPC.

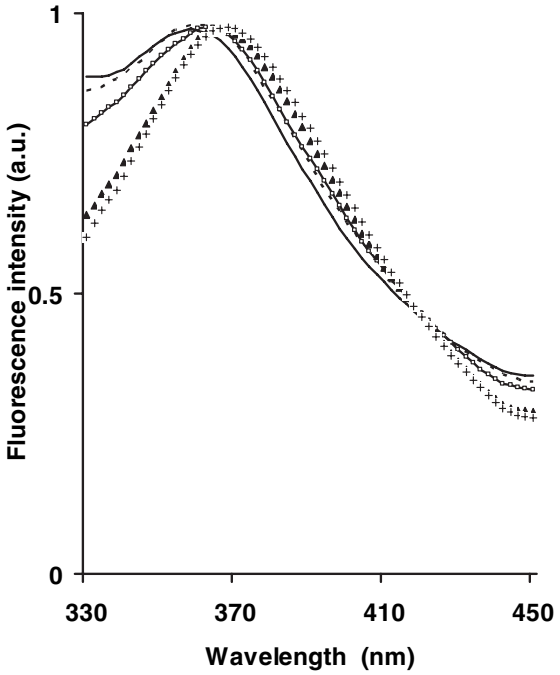

Figure 1. Normalized ubiquinol fluorescence emission spectra in EggPC unilamellar liposomes. Ubiquinol 1 mol.% (——), 2 mol.% (- - -), 3.2 mol.% (–□–), 10 mol.% (▲), 20 mol.% (+).

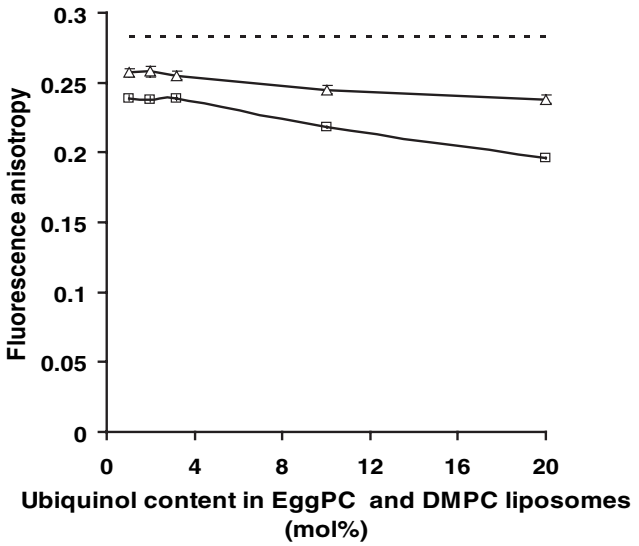

Figure 2. Fluorescence anisotropy of ubiquinol in EggPC (□) and DMPC (Δ) at different ubiquinol contents measured at 30°C; (- - -) stands for ubiquinol maximal anisotropy determined in immobilized matrices (10).

Table 1. Percentage of ubiquinol-10 (UQH_2) oxidation by H_2O_2 in egg phosphatidylcholine small unilamellar liposomes.

[UQH_2] mol.%	% UQH_2 oxidation
3.2	15
10	10
20	8

At low UQH_2 mol.% (up to 3.2 mol.%) similar values of r_s (almost close to UQH_2 fundamental anisotropy value) (10) are observed in both phospholipid liposomes without significant changes. Increasing UQH_2 content, UQH_2 r_s values show a slight, but significant, decrease.

UQH_2 fluorescent lifetime in DMPC liposomes was best fitted by a biexponential decay with a major component of 0.4 ns (data not shown). In Table 1 the oxidation percentage of UQH_2 by 50 mM H_2O_2 in EggPC small unilamellar liposomes is reported. The UQH_2 oxidation is followed measuring the decrease in absorption at 290 nm. We observe a low value of oxidation percentage and a significant oxidation decrease with increasing UQH_2 concentration.

Steady-state Laurdan excitation and emission spectra

In control experiments the spectral characteristics of the probe were unaltered by the addition of UQH_2, indicating the lack of a direct interaction between the two compounds.

Normalized excitation and emission spectra of Laurdan in EggPC LUVs without and with 10 and 20 mol.% UQH_2 are reported in Fig. 3A,B, respectively. The measurements were performed at 23°C and at this temperature EggPC is in the fluid state. Laurdan excitation and emission spectra are indicative of a liquid–crystalline phase—the excitation spectra displays two peaks, one at ~350 nm and the other at ~390 nm, and the ratio of 390–350 nm band is <1, while the emission maxima are observed at 456 nm. In the presence of UQH_2 10 and 20 mol.% the excitation spectra show the ratio of 390–350 nm band >1 and are redshifted while the emission spectra are blueshifted; UQH_2 3.2 mol.% has no effect (data not shown).

Fluorescence excitation and emission maxima values of Laurdan in DMPC LUVs at 31°C with and without 20 mol.% UQH_2 are reported in Table 2. At this temperature the phospholipids are in the liquid–crystalline phase and 20 mol.% UQH_2 induces slight, but significant blueshift in the excitation and emission spectra. No significant changes in excitation and emission spectra were observed with UQH_2 3.2 and 10 mol.% (data not shown).

Laurdan generalized polarization

The wavelength dependence for Laurdan excitation (Ex GP) and emission (Em GP) parameters are measured in EggPC LUVs in the absence and in the presence of UQH_2 2, 3.2, 10, 20 mol.% at 23°C (Fig. 4A,B). In EggPC without UQH_2 the Ex GP values decrease with increasing excitation wavelength while the Em GP values show an increase with increasing emission wavelength. This behavior is characteristic of phospholipids in the liquid–crystalline phase. The presence of

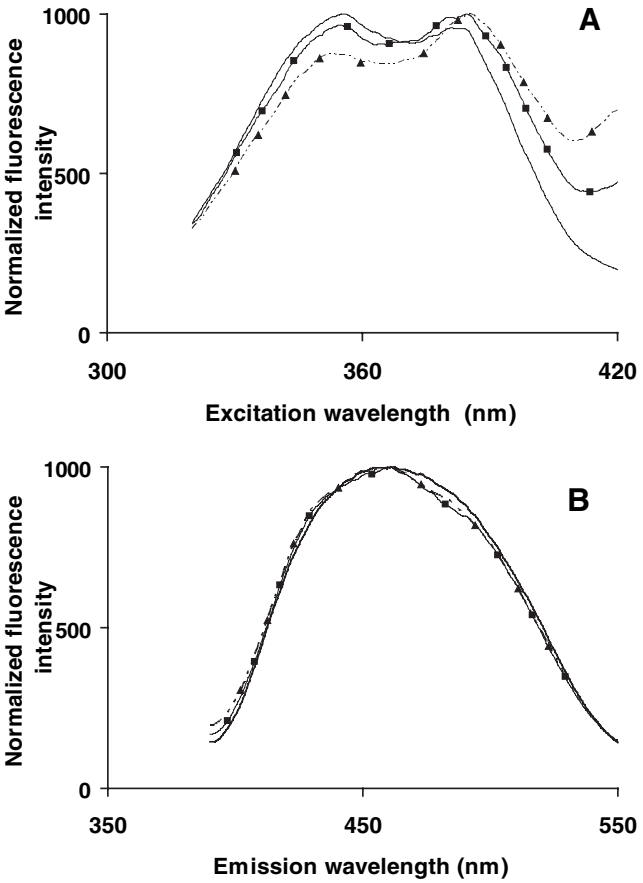

Figure 3. Laurdan excitation (A) and emission (B) spectra in EggPC LUVs. Control (——), ubiquinol 10 mol.% (–■–), 20 mol.% (–▲–). Laurdan fixed emission and excitation wavelengths are 430 and 360 nm for excitation and emission spectra, respectively.

Table 2. Excitation and emission maxima of Laurdan in DMPC LUVs at 31°C with and without UQH_2.

	F_{max} (nm)	
	Excitation	Emission
DMPC	357	444
DMPC + 20 mol.% UQH_2	353	442

DMPC = dimyristoyl phosphatidylcholine; LUVs = large unilamellar vesicles; UQH_2 = ubiquinol-10.

UQH_2 induces biphasic Ex GP and Em GP wavelength dependence pattern which is characteristic of two coexisting phases. In the Ex GP the biphasic pattern is evident at 10 and 20 mol.% UQH_2, while in the Em GP it starts to be evident at the lowest UQH_2 concentration.

The wavelength dependences for Laurdan Ex GP and Em GP in DMPC LUV_S in the absence and in the presence of 3.2, 10, 20 mol.% $CoQH_2$ at 31°C are shown in Fig. 5A,B, respectively. At this temperature, a typical wavelength dependence of Laurdan excitation and emission spectra indicates a liquid–crystalline phase. The presence of 10 and 20 mol.% $CoQH_2$ modifies the spectral appearance—the Ex GP values increase above 400 nm; the Em GP values decrease between 420 and 440 nm, leading to a negative slope, and then increase

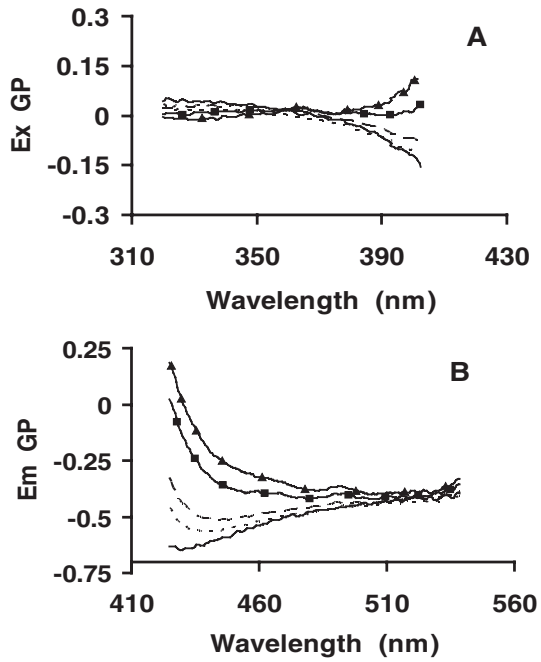

Figure 4. Laurdan excitation (A) and emission (B) GP spectra in EggPC LUVs with different ubiquinol concentrations. Control (——), ubiquinol 2 mol.% (·····), 3.2 mol.% (- - -), 10 mol.% (–■–), 20 mol.% (–▲–).

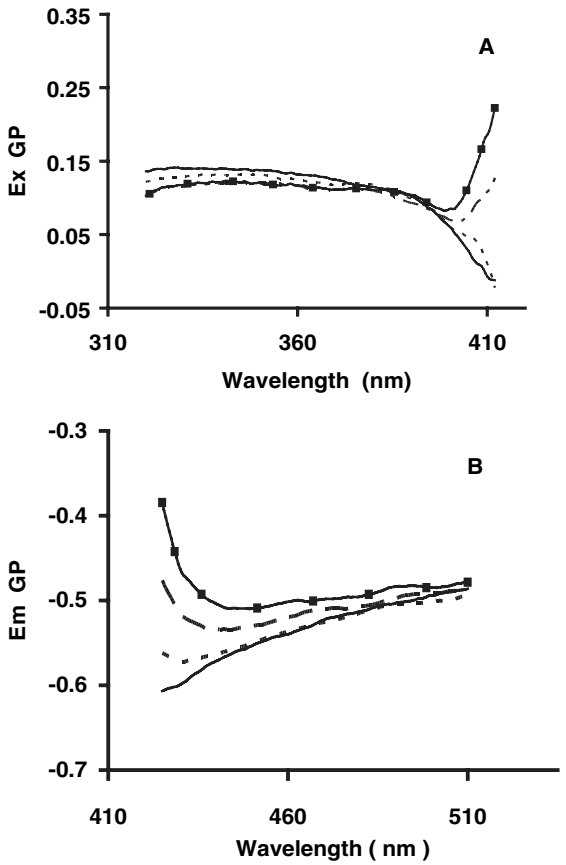

Figure 5. Laurdan excitation (A) and emission (B) GP spectra in DMPC LUVs at 31°C with different ubiquinol concentrations. Control (——), ubiquinol 3.2 mol.% (·····), 10 mol.% (---), 20 mol.% (–■–).

in a similar manner to the control, but with higher values. The lowest concentration of $CoQH_2$ (3.2 mol.%) affects very slightly both the GP spectra.

DISCUSSION

The location of UQH_2 in the biologic membranes is still a matter of discussion and its interaction with the phospholipid bilayer is not completely understood. Many physical techniques have been used to study UQ (the oxidized form) locations but corresponding studies of hydroquinones are limited. UQ would lie parallel to the membrane plane in the bilayer center (19–21) where it could also exist in the form of aggregates interdispersed among the hydrocarbon tails of phospholipids (22). In contrast, other researchers using calorimetric techniques have suggested that the UQH_2 ring (the reduced form) interacts with the phospholipid polar heads with the polyisoprene domain anchored to the hydrophobic bilayer (23). The fluorescence properties of plastoquinol, ubiquinol and α-tocopherol in solution and in model membranes have been characterized (24). Recently, the intrinsic fluorescence of plastoquinols and α-tocopherol has been used to study their orientation and dynamics in model membranes (10).

UQH_2 exhibits negligible fluorescence in aqueous solutions, while in EggPC liposomes it shows a fluorescence emission spectra with a maximum that is redshifted by increasing ubiquinol concentrations. This shift could depend on changes in its fluorescence quantum efficiency which is affected by the micropolarity of the environment where it is located (24).

UQH_2 steady-state fluorescence anisotropy (r_s) does not change very much up to 3.2 mol.% UQH_2 and as its fluorescence efficiency is much higher in hydrophobic than in polar solvents (24), we should measure the r_s of UQH_2 molecules located in the membrane interior. This is not in agreement with the high r_s and the short lifetime values we have found in fluid liposomes, indicating that UQH_2 has a distribution much closer to the polar water-lipid interface. This finding seems in accordance with the calculations made by Kruk *et al.* (24) who have considered the fluorescence efficiency and the molar fractions of UQH_2 molecules in polar and hydrophobic regions of liposome membranes. The small but significant decrease in r_s at concentrations of 10 and 20 mol.% could reflect a slightly deeper UQH_2 location in the bilayer at increasing concentrations. These results are supported by the oxidation experiments performed in the same sample used for the r_s measurements where we observed a decrease in oxidation with increasing UQH_2 concentration.

Recent experimental evidence (25), by using NMR chemical shift–polarity correlation, has shown that the hydroquinone ring is distant from the lipid–water interface. In order to obtain a further insight into the interaction between UQH_2 and the bilayer, Laurdan fluorescence spectroscopy was utilized. Laurdan shows no preferential phase partitioning between ordered and disordered lipid phases, does not have specific affinity toward any phospholipid head group and is believed to have uniform lateral and transbilayer distribution (26). Therefore we can conclude that the effects of UQH_2 on the fluorescence properties of Laurdan in EggPC and DMPC are probably due to changes in the microenvironment of the probe and not to changes in probe localization. Its fluorescent naphthalene ring is located at the hydrophilic–hydrophobic

interface of the phospholipid bilayer, at ~5 Å from the membrane surface (27) and its emission spectra maxima depend both on the polarity of the environment surrounding the probe and on the rate of relaxation of water molecules, or molecular residues, that can reorient around Laurdan fluorescent moiety during its excited state lifetime (28). In gel phase membranes the rate of water molecule dipolar relaxation is too slow to affect fluorescence emission, while in LC phase the dipolar relaxation occurs during the fluorescence lifetime, inducing a redshift of light emission. Thus, the emission spectral shifts we have observed and quantified by GP in EggPC and DMPC LUVs could reflect changes in the bilayer polarity (water content) and/or changes in dipolar relaxation (mobility of adjacent water molecules). Changes in polarity cause the emission and excitation spectra to shift in the same directions while changes in dipolar relaxation cause the emission and the excitation spectra to shift in opposite directions (28). Our results show that ubiquinol affects the molecular dynamics at the hydrophilic/hydrophobic membrane interface of EggPC and DMPC LUVs in a different way. In EggPC UQH_2 caused the spectra to shift in opposite directions. The excitation spectrum is redshifted while the emission spectrum shows a blueshift reflecting a decrease in the rate of dipolar relaxation of water molecules near the probe as has been described for cholesterol effect in liposomes in the LC phase (28). In DMPC in the LC phase, UQH_2 caused both the spectra to have a blueshift, reflecting a decrease in polarity around the fluorescence moiety of the probe, that means a decreased hydration of the bilayer.

The excitation spectra of Laurdan in membranes displays two peaks—one at ~360 nm and the other at ~390 nm. The band centered at ~390 nm is a distinct feature of Laurdan spectroscopy and its intensity depends both on the polarity of the probe environment and also on the phase state of phospholipids. For Laurdan in gel-phase phospholipids this red excitation band also constitutes the excitation maximum, while in the LC phase it is less intense (29). In EggPC LUVs it displays two peaks and the ratio of the intensities of the peaks (390:360) is < 1 indicating the LC phase. In the presence of ubiquinol the ratio becomes > 1, indicating a gel phase which is caused most likely by the ubiquinol-induced increase in lipid molecule packing.

Laurdan spectral shifts are usually quantified in the form of GP. Useful information about the membrane state is obtained by utilizing the wavelength dependence of Laurdan GP spectra (29). A wavelength-independent GP spectrum is characteristic of the gel phase, while in liquid–crystalline phases the GP spectrum typically displays wavelength dependence, due to the dipolar relaxation process; in particular the Ex GP values decrease with increasing excitation wavelength while the Em GP values show an increase with increasing emission wavelength. In the case of two coexisting phases, the GP spectrum shows the opposite trend (*i.e.* the excitation GP values increase with increasing excitation wavelengths and the emission GP values show a reduction with increasing emission wavelengths). Ex GP in liquid–crystalline phase mainly reports the rate of dipolar relaxation (29), while Em GP reports the polarity of the probe environment (28,30). Thus, if the lipid molecules surrounding the probe become more densely packed, both polarity and dipolar dynamics decrease and are reported as increased Em GP and Ex GP, respectively.

The GP values and also the wavelength dependence of the excitation and emission GP spectra of Laurdan in EggPC LUVs imply the presence of the liquid–crystalline phase. Increasing concentrations of UQH_2 cause a significant increase in Ex GP in the red excitation band and an increase in Em GP in the blue emission band. These findings mean that in liquid–crystalline membranes the presence of UQH_2 induces an increase in Laurdan molecules surrounded by phospholipids much more packed, thus inducing the coexistence of gel and liquid–crystalline domains. UQH_2 induces the same GP pattern in DMPC LUVs in LC phase with a low but always significant effect. In EggPC LUVs the effects are much more evident and this different behavior could be due to a different amount of water adsorbed (31). Importantly the GP value is independent of the chemical nature of the phospholipid head group (15), but it is greatly affected by the local membrane packing that allows water to go into the membrane and to relax around the Laurdan naphthalene group.

Based on the data presented here, the ubiquinol ring is not segregated to the center of the bilayer, but it locates near the polar head group of the phospholipids where its hydroxyl groups could form hydrogen bonds with water, increasing the packing of the phospholipids.

Acknowledgements—T.L.H. and E.G. supported by NIH (PHS 5 P41-RR003155) and UCI.

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