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A study of quercetin effects on phospholipid membranes containing cholesterol using Laurdan fluorescence

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Abstract Quercetin (QCT) is an important bioactive natural compound found in numerous edible plants. Since the lipid bilayer represents an essential compound of the cell membrane, QCT's direct interaction with this structure is of great interest. Therefore, we proposed to study the effects of QCT on DMPC liposomes containing cholesterol (Chol), and for this purpose Laurdan fluorescence was used. As a fluorescent probe, Laurdan is able to detect changes in membrane phase properties. When incorporated in lipid bilayers, Laurdan emits from two different excited states, a non-relaxed one when the bilayer packing is tight and a relaxed state when the bilayer packing is loose. The main tool for quantifying QCT's effects on phospholipid membranes containing Chol has been the analysis, the decomposition of Laurdan emission spectra in sums of two Gaussian functions on energy. This kind of approach has allowed good analysis of the balance between the two emitting states of Laurdan. Our results show that both Laurdan emission states are present to different extents in a wide temperature range for DMPC liposomes with Chol. QCT is decreasing the phase transition temperature in pure DMPC liposomes as proved by generalized polarization (GP) values. QCT also quenches Laurdan fluorescence, depending on the temperature and the presence of Chol in the membrane. Stern-Volmer constants were calculated for different lipid membrane compositions, and the conclusion was that the relaxed state favors the nonradiative transitions of the fluorophore.

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Introduction

The lipid bilayer plays an active role in all processes that occur in the cell life cycle, directly interacting with all types of compounds. Among the wide range of categories of compounds that interact with the lipid membranes, antioxidants are of great interest. We chose to study the effects of the antioxidant quercetin (QCT), a bioactive natural compound belonging to the class of flavonoids that can be found in the human daily diet. Widely spread sources of QCT include green tea, apples, grapes, berries, cherries, red onions, broccoli and cocoa. Flavonoids are generally known to protect the human body against environmental stress, and they are believed to promote cellular survival through modulation of intracellular signals. QCT is supposed to have beneficial effects on the cardiovascular and ophthalmologic systems, and it has anti-inflammatory behavior. Psahoulia et al. (2007a) showed that, in nontoxic concentrations (less than 20 μM), QCT inhibits the proliferation of malignant cells, at least by selective induction of oncogene Ras protein forms of degradation. It also potentiates induced apoptotic death (Psahoulia et al. 2007b; Kim and Lee 2007), and some studies indicate that QCT acts as an adjuvant in chemotherapy (Thangasamy et al. 2007; Baran et al. 2011).

Despite the many studies concerning QCT's influence on cell metabolism, the mechanisms of its functioning have not yet been completely elucidated. Movileanu et al. (2000) showed that the antioxidant activity of QCT depends on its direct interaction with the lipid bilayer of the cell membrane; QCT insertion in the membranes is concentration- and

pH-dependent, the interaction of the flavonoid with the membrane having no impact on the structure or on the integrity of the lipid bilayer. The mechanism of QCT's action on cell membranes, not only by its interaction with proteins, but also through its interaction with the phospholipid bilayer, is not fully understood (Ionescu et al. 2007).

Here we are interested in the interaction of QCT with pure lipid bilayers in the presence of cholesterol (Chol). We explored QCT's effects on phospholipid bilayers containing Chol using a spectrofluorimetric approach. We used Laurdan (2-dimethylamino-6-lauroylnaphthalene) as a fluorescent probe because it shows a high sensitivity to environment polarity and therefore can detect changes in the membrane's state of aggregation. Laurdan's fluorescence properties in lipid membranes have been attributed to the presence of water molecules at the different bilayer phases at the location of the fluorophore (Parasassi et al. 1991). Two of the most important factors that influence the rate of solvent relaxation are known to be the temperature and viscosity of the solvent (Lakowicz 2006). When incorporated in the lipid bilayers, Laurdan emits from two different excited states, a non-relaxed state when the bilayer packing is tight and a relaxed state when the bilayer packing is loose. Because lipid packing is highly dependent on temperature, this behavior of the fluorophore is noted in its fluorescence emission spectra as a shift of maximum emission fluorescence from 440 nm at temperatures below phase transition (when the lipids are in the gel phase) to 490 nm at temperatures above the phase transition values (when the lipids are in the fluid phase). Hence, Laurdan can be used for monitorizing the lipid order (Parasassi et al. 1991).

The fluorescence emission spectra of Laurdan were analyzed as a sum of two Gaussian bands, centered on the short emission wavelength and on the long emission wavelength, respectively, corresponding to the two excited state populations, thus allowing a very good evaluation of the relative presence of each population. This kind of decomposition has been suggested by the shape of the fluorescence emission spectra, especially in the case of the ones recorded from the probes containing Chol. The decomposition was done as a function of energy (Lucio and De Vequi-Suplicy 2010) and proved to be an excellent tool for analyzing the balance between a non-relaxed (Laurdan molecules inserted in the gel phase of the lipid membrane) and a water-relaxed emission population (Laurdan molecules inserted in the liquid-crystal phase of the lipid membrane) (De Vequi-Suplicy et al. 2006).

We developed a series of experiments in order to see how QCT influences the phase segregation of DMPC liposomes with/without Chol. Its insertion into the lipid bilayers leads to a decrease of phase transition temperature in the pure phospholipid membrane, as shown by Laurdan emission generalized polarization (GP) (Parasassi et al. 1990) and also by the dependence of fraction areas on temperature as calculated by the decomposition of the spectra in two Gaussian bands.

Another result of our experiments proves that QCT acts as a quencher of Laurdan fluorescence. Quenching of fluorescence requires a close approach of the fluorophore and quencher. From quenching parameters one should obtain information regarding the relative positioning of the two types of molecules involved in the quenching process. Usually, quenching is supposed to be either dynamic or static, or a combination of both of them (Lakowicz 2006), and those types of approaches are successful in studies implying isotropic and low-viscosity solutions. In these particular cases, according to the Stern-Volmer theory, the ratio of fluorescence intensities, in the absence and in the presence of the quencher, depends linearly on quencher concentration. Due to the anisotropic character of lipid membranes, their components are constrained to two-dimensional movements and have lower diffusion rates than in non-viscous solvents.

In our case, the fluorescence intensities ratio showed an exponential dependence on quencher concentration; therefore, we used a modified Stern-Volmer equation (Lakowicz 2006). We fit the dependence of the ratios of fluorescence intensities on the quencher concentration, in the absence and in the presence of the quencher, with an exponential function. Using the fit parameters we calculated the distance to which the two compounds approach while temperature increases, and we found this value to be 15 ± 3 Å independent of temperature or QCT concentration.

Materials and methods

For the steady-state fluorescence measurements, liposomes (small unilamellar vesicles SUV) were prepared from 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (14:0 PC) (DMPC) (Sigma). The buffer was PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH = 7.4; all substances were ≥99%, from Sigma). QCT, purity 98% HPLC (Sigma), was dissolved in dimethyl sulfoxide (DMSO, ≥99.7% from Sigma) in 1 mM or 10 mM stock solution, used in such a manner that the DMSO concentration in the cuvette did not exceed 1%. Preliminary experiments showed that DMSO had no effects whatsoever on the recorded spectra in the concentrations used during our experiments. For SUV preparation, a known amount of DMPC was weighed and dissolved in chloroform (≥99% from Sigma); the mixture was dried in nitrogen atmosphere under continuous rotation and then resuspended in PBS. The suspension was then ultrasonicated and diluted with PBS to a value of absorbance of 0.3, which corresponded to

a final 0.2 mM lipid concentration in the probe. Liposomes containing Chol were prepared using the same procedure as for those with no Chol, but the final lipid mixture contained 20% or 33% Chol (Sigma). Aliquots of 2.5 ml from the suspension were prepared and kept at −20°C until use. Before each experiment, the liposomes were thawed at room temperature and ultrasonicated.

The 2-ml liposome suspension samples were placed in a quartz cuvette with an optical pathway of 10 mm. Laurdan (2-dimethylamino-6-lauroylnaphthalene; Molecular Probes) dissolved in DMSO was added at 1 μM concentration in order to keep the concentration of the organic solvent low enough to avoid altering the biological samples. The suspension was continuously stirred with a small magnet. For fluorescence emission measurements, we used a HORIBA Jobin-Yvon Fluorolog 3-11 (FL3-11) with a xenon lamp. The excitation and emission slits were set in the range of 0.95–1.5 nm, all the data being recorded with adequate correction, averaged and normalized. Appropriate blanks corresponding to the liposome suspension in buffer without Laurdan were subtracted to correct the background fluorescence. Personal unpublished data revealed that, in our experimental conditions, QCT does not absorb 440-nm wavelength radiation and does not present fluorescence when excited at 440 nm, in agreement with data from the literature (Mezzetti et al. 2011).

The samples were thermostated using a Peltier Wavelength Electronics LFI-3751, and the temperature varied from 1 to 52°C, with a 2°C or 3°C step. The emission spectra were obtained with the excitation wavelength at 364 nm. Preliminary experiments showed that the energy corresponding to this wavelength is not absorbed by QCT. Laurdan emission spectra were decomposed in two Gaussian bands using the software *OriginPro7.5*. All the spectra were normalized to the values recorded at 10°C for each experiment.

Results and discussions

Effect of Chol on DMPC liposomes

In our first type of experiment, we recorded the fluorescence emission spectra of Laurdan incorporated in DMPC/(DMPC + 20% or 33% Chol) liposomes when temperatures varied within an interval centered on the phase transition temperature (4–50°C). All the spectra were normalized at the values recorded at 10°C. The maximum Laurdan fluorescence emission moved from 440 nm at low temperatures (below the main phase transition of the lipids) to 490 nm at high temperature in the case of pure DMPC liposomes (Fig. 1a). However, when Chol was present (20%), such a shift of the maximum emission intensity was not that obvious (Fig. 1b) (similarly for 33%, results not shown). For all recorded spectra the amplitude of the fluorescence signal decreased to approximately 0.6 at 40°C.

The spectra of Chol containing liposomes presented an isoemissive point at 487 nm (see Fig. 1b) for liposomes with 20% Chol (similarly for 33%, results not shown). The isoemissive point shows the temperature dependence of the interconversion of two different populations of Laurdan molecules (Chapman et al. 1995). In the case of DMPC liposomes such an isoemissive point was apparently absent for the entire range of temperatures. But, if only the spectra recorded below phase transition temperatures were analyzed (Fig. 2a), an isoemissive point appeared even for pure DMPC liposomes at an emission wavelength of 479 nm. Since the isoemissive point was present in the gel phase of pure DMPC membranes and in DMPC membranes containing Chol, the disappearance of the isoemissive point was clearly related to the larger fluidity of the pure lipid membrane above its main transition temperature.

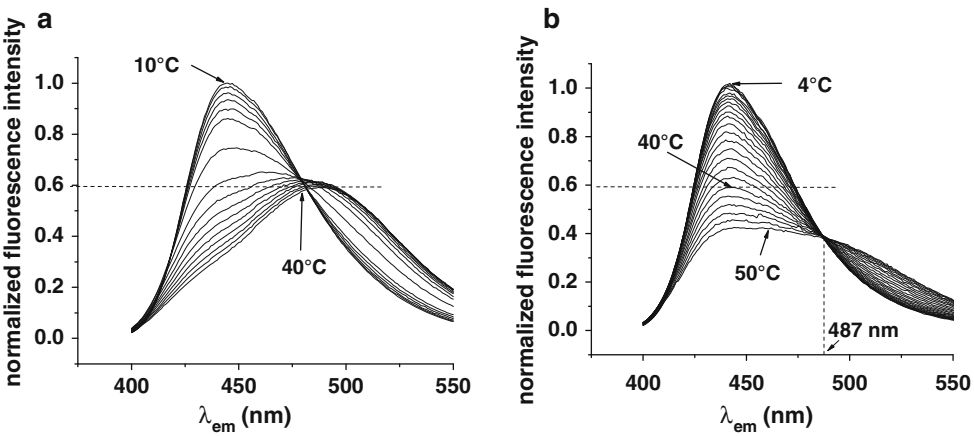

Fig. 1 Laurdan emission spectra from DMPC liposomes in PBS at different temperatures (**a**) and from DMPC liposomes with 20% Chol (**b**) (similarly for 33% Chol, results not shown)

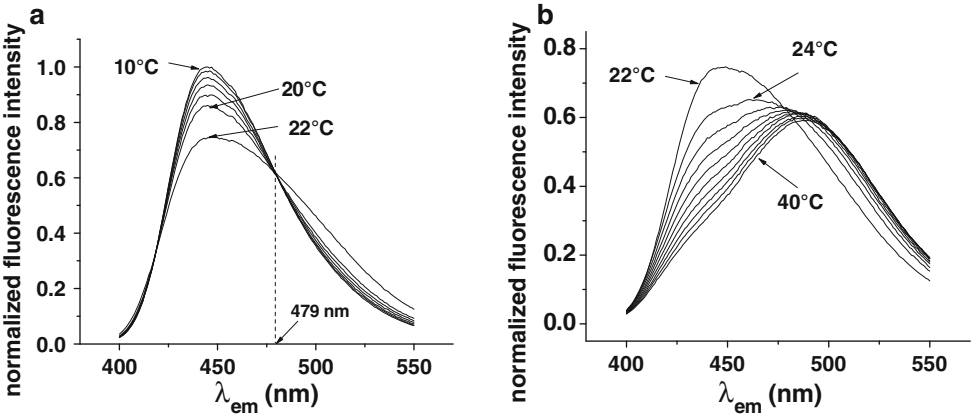

Fig. 2 Laurdan emission spectra from DMPC liposomes in PBS below phase transition temperatures (**a**) and above phase transition temperatures (**b**)

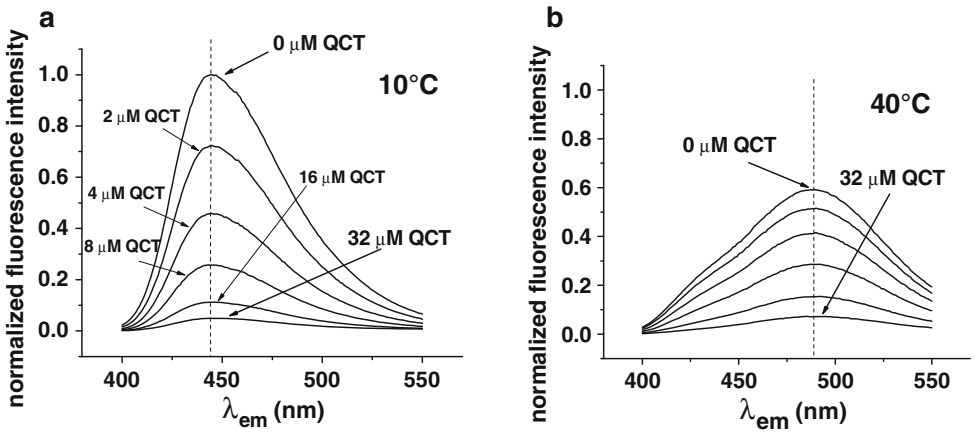

Fig. 3 Comparison of Laurdan fluorescence emission spectra at 10°C (**a**) and 40°C (**b**), with increasing QCT concentrations, for pure DMPC liposomes. The values are normalized at the maximum value recorded at 10°C in the absence of QCT

Effect of QCT on DMPC liposomes in the absence and presence of Chol

When QCT was added, our first observation was that the amplitudes of Laurdan emission fluorescence spectra decreased, but the emission wavelengths were not modified (Figs. 3, 4).

The diminution in signal amplitude demonstrated that QCT acts as a quencher on Laurdan fluorescence, including the cases when the fluorescent probe was incorporated inside the membrane of liposomes containing Chol, in the entire range of studied temperatures. This effect has been observed before in DMPC liposomes marked with chlorophyll a (Chla) (Dragusin et al. 2010).

It can also be observed that in pure DMPC liposomes the signal amplitudes at lower temperatures decay faster as compared to those at high temperatures (Fig. 5a). The quenching is more accentuated on Laurdan fluorescence emitted from the non-relaxed state than from the relaxed one. Quenching supposes the close proximity of the quencher and the fluorophore molecule. In our case, both Laurdan and QCT have a hydrophobic character and therefore are supposed to be found inside the membrane core. At low temperatures, when lipid packing is tight, water molecules from membrane regions where Laurdan molecules reside are not that numerous, and QCT quenches Laurdan fluorescence with a higher efficiency. At high temperatures, where the lipid membrane is in the liquid-crystal phase and lipid packing is loose, the amount of water in the vicinity of Laurdan molecules is large, and QCT molecules are probably repelled. Therefore, the quenching becomes less efficient. In fact, Laurdan fluorescence intensity decreases as the temperature increases from 10 to 22°C, with its value at 22°C being the lowest one. The temperature value of 22°C is the one for the main phase transition for DMPC. Then, Laurdan's fluorescence intensity increases with increasing temperature, and its highest value is recorded at 40°C (Fig. 5a).

When liposomes contained Chol (Fig. 5b), at high temperatures the quenching was more accentuated than in the case of low temperatures. The highest amplitude value appears to be found at 22°C (this temperature is close to the phase transition temperature for DMPC). The intensity of fluorescence emitted by Laurdan increases with temperature up to 22°C and then decreases, with the lowest value being recorded for the highest temperature (52°C). The decrease of the fluorescence intensity at high temperatures shows that Chol keeps an order in the membrane structure

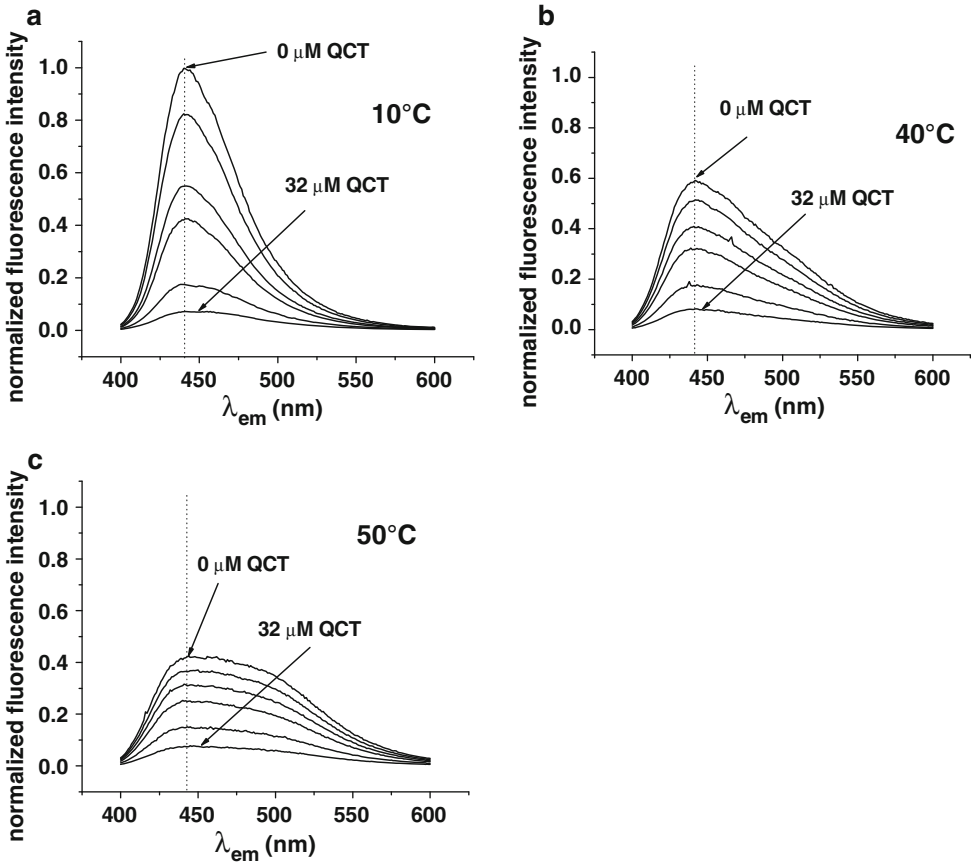

Fig. 4 Comparison of Laurdan emission spectra at 10°C (**a**), 40°C (**b**) and 50°C (**c**), with increasing QCT concentrations for liposome DMPC containing 20% Chol. The values are normalized at the maximum value recorded at 10°C in the absence of QCT (in the same concentrations as in Fig. 3a)

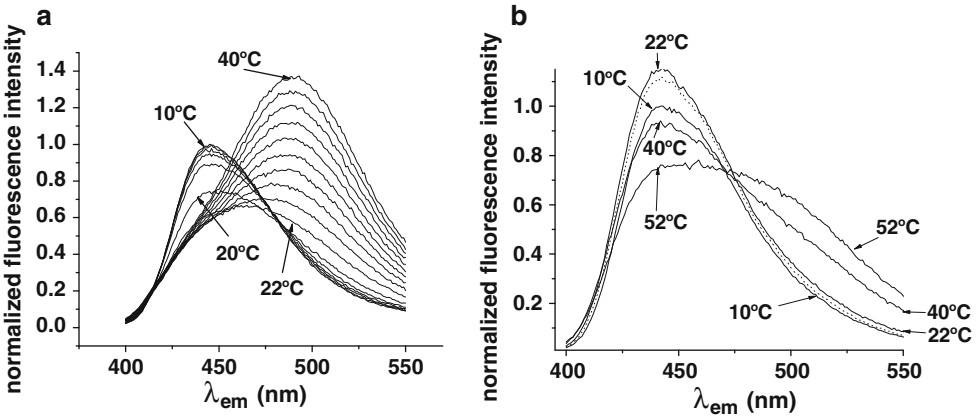

Fig. 5 Laurdan fluorescence emission spectra for pure DMPC liposomes (**a**) and for liposomes containing 20% Chol (**b**) in the presence of 16 μM QCT (*dotted line*, 16°C) (similarly for 33% Chol liposomes, not shown)

that allows QCT to approach Laurdan molecules in an easier manner, similarly to the case of pure DMPC liposomes at low temperatures (Fig. 5a). Actually, even at high temperature, Chol expels water molecules. The isoemissive point may still be seen in the presence of QCT, but it shifted from 487 nm when no QCT was added to 473 nm (Fig. 5b).

As shown in Fig. 5a, in the temperature interval from 10 to 22°C, quenching is higher than in the interval from 22 to 40°C because of the competition of the fluorescence emission, non-radiative decay and relaxation.

Therefore, Laurdan fluorescence quenching by QCT depends not only on temperature, but also on the presence of Chol in the membrane.

Effect of QCT on the phase transition temperature of DMPC liposomes in the absence and presence of Chol

In order to quantify QCT effects on phospholipid membranes, we found the phase transition temperature using the generalized polarization (GP) (Parasassi et al. 1990).

The dependence on phase transition temperature on QCT concentration has been fitted with an exponential decay (Fig. 6), showing that the diminution of lipid membrane phase transition temperatures (Saija et al. 1995) occurs faster at lower QCT concentrations.

For liposomes with Chol, GP did not reveal any information regarding the phase transition temperature of the lipid bilayers, and the fit with a sigmoid function had given huge errors of about 100%. Therefore, we calculated the phase transition temperatures in different conditions, using the decomposition of all the recorded spectra in sums of two Gaussian bands. This approach was suggested with the predilection by the shape of Laurdan emission spectra recorded in the case of Chol containing liposomes at high temperatures (Fig. 7b). We fitted the normalized fluorescence emission intensities $\Im(\lambda) = \left(\frac{I_{t^\circ C}}{I_{10^\circ C}}\right)$ for all the spectra, with the sum of two Gaussian functions of wavenumber $v = 1/\lambda$ (Lucio and De Vequi-Suplicy 2010):

$$\tilde{\Im}(v) = I_{\text{Blue}} \cdot \exp\left(-\frac{\left(v - \frac{1}{\lambda_{\text{Blue}}}\right)}{\sigma_{\text{Blue}}} \cdot\right)^2 + I_{\text{Red}} \cdot \exp\left(-\frac{\left(v - \frac{1}{\lambda_{\text{Red}}}\right)}{\sigma_{\text{Red}}} \cdot\right)^2 \quad (1)$$

where we used the relation $\Im(\lambda) = \tilde{\Im}(v)\frac{\mathrm{d}v}{\mathrm{d}\lambda}$ (Lakowicz 2006) and the following free parameters: maximum amplitudes of the Gaussians (I_{Blue}, I_{Red}) and their corresponding positions (wavelengths λ_{Blue} and λ_{Red}, which proved to be around 440 and 490 nm, respectively), as well as their bandwidths (σ_{Blue}, σ_{Red}).

By analyzing the decomposition (Fig. 7a) one can see that, even at very low temperatures (10°C), a considerable contribution of the Laurdan fluorescence emission is given from the relaxed state, while at higher temperatures, the contribution of the "blue" band decreases substantially (not shown), meaning that both states are present with different shares at all temperatures, as has been found before (Lucio and De Vequi-Suplicy 2010).

For low temperature, for liposomes containing Chol (not shown), the contributions of the two Laurdan emission states appear to be almost equal, similarly to the case of pure DMPC liposomes. At 10°C, for all studied liposomes, the amplitude of Laurdan fluorescence emission from the relaxed state is almost equal to the amplitude of its emission from the non-relaxed one. This means that half of Laurdan radiative deexcitation originates from its relaxed excited state, even for Laurdan molecules distributed in the gel phase of the lipids. At 40°C, for pure DMPC, the total amplitude of Laurdan fluorescence emission decreases, but only because of the contribution from the non-relaxed Laurdan emission state (from 0.6 at 10°C to 0.1 at 40°C), while the contribution from the relaxed state is constant (0.6) and becomes predominant. When Chol is part of the liposome, at 40°C, although the total fluorescence emission is 60% from the total emission at 10°C, similarly to the case of pure DMPC liposomes, the contributions of the two Laurdan-emitting states are equal (not shown). At higher temperature (Fig. 7b), the amplitude of the total fluorescence emission intensity decreases only because of the diminution of the emission from the non-relaxed Laurdan state.

For all types of liposomes the center of the Gaussians showed variations with temperature (Fig. 8a, b). The augmentation of λ_{red} with temperature may be accounted for by the fact that increasing temperature leads to a looser packing of the Laurdan molecules' environment, and hence, Laurdan fluorescence emission is produced from an even more relaxed state. Although the two emitting wavelengths seem to depend sigmoidally on temperature, this fit did not reveal any information on phase transition temperature.

Therefore, temperature values for the lipid phase transition were calculated instead by plotting the fraction areas of the Gaussians versus temperature (tagged "Blue" and "Red" in Fig. 9). All the curves were fitted with sigmoid functions. This kind of fit gave very small errors for all the types of liposomes in the presence of QCT in a large concentration range; it allowed the extraction of the values for phase transition temperatures and proved that the binary system DMPC-Chol appears nonhomogenous on a wide temperature scale.

For pure DMPC liposomes, the phase transition temperature decreased when the QCT concentration increased (also revealed by GP). When Chol is part of the liposomes, in different concentrations, there is no notable effect of QCT on phase transition temperature (Fig. 6). For 33% Chol containing liposomes the phase transition temperature

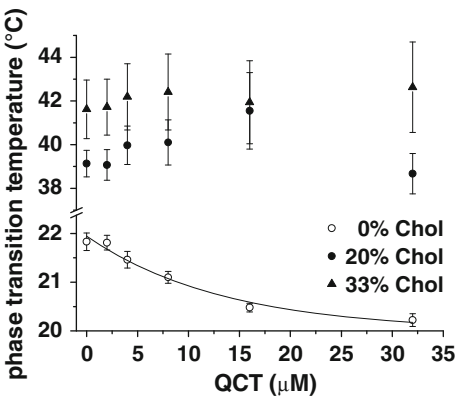

Fig. 6 Phase transition temperature dependence on QCT concentration (for liposomes containing Chol, phase transition temperature could not be calculated using GP values)

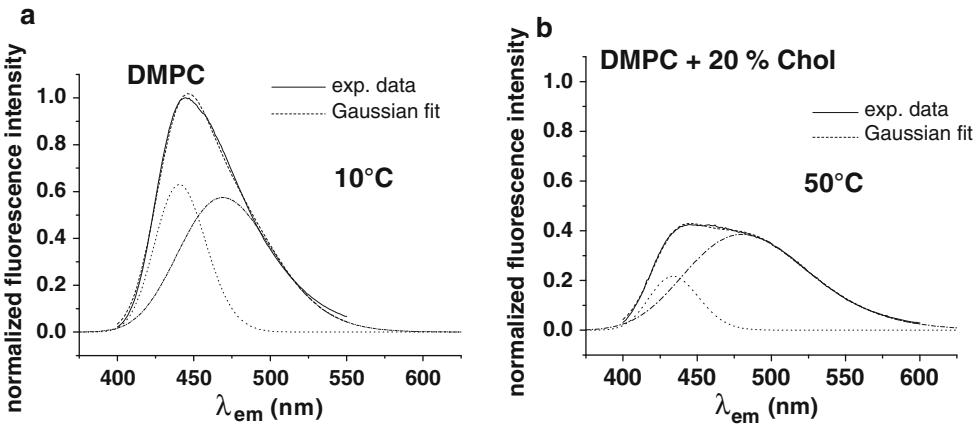

Fig. 7 Typical decomposition of Laurdan fluorescence emission spectra into two Gaussian functions for liposomes containing different concentrations of Chol (no Chol **a**; 20% Chol **b**); for 33% Chol, the results are similar to the ones for 20% Chol, not shown) at different temperatures

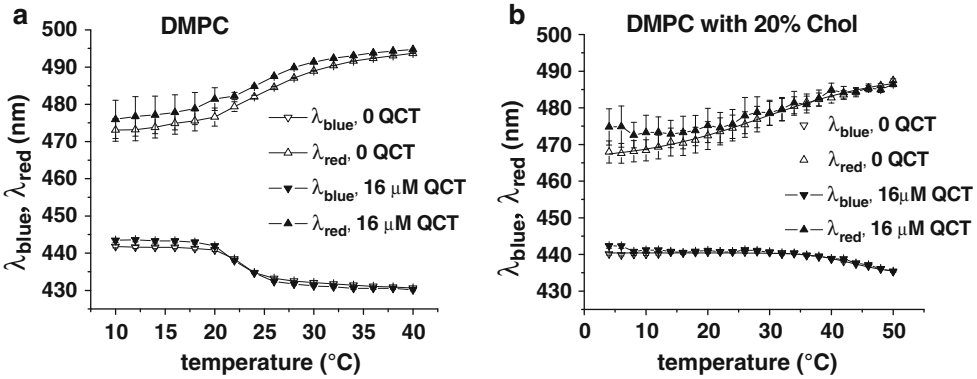

Fig. 8 The dependence on temperature of the short and long wavelengths on which the two Gaussian bands are centered for DMPC liposomes with different concentrations of Chol

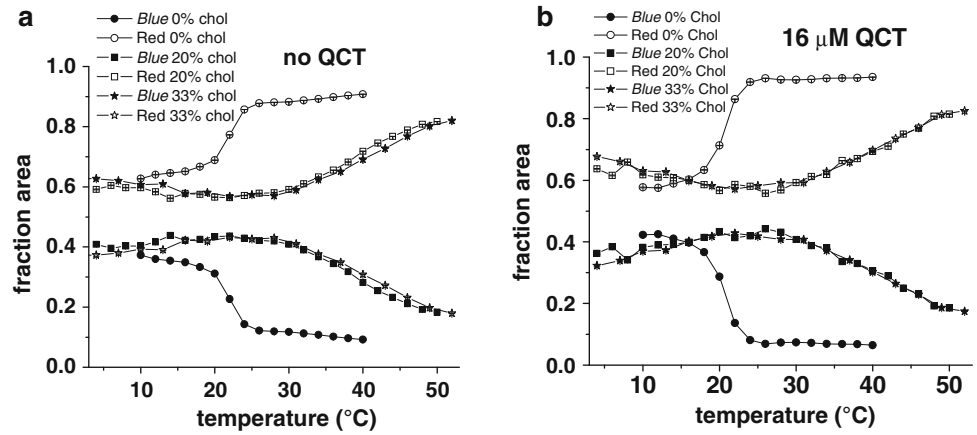

Fig. 9 Fraction areas of the two Gaussian functions with no QCT (**a**) and 16-μM QCT (**b**) are plotted versus temperature for liposomes containing different Chol concentrations

is situated around 42°C, while for liposomes with less Chol, this value is situated around 40°C. The cumulative contribution of QCT and Chol keeps the phase transition temperature of Chol containing liposomes at an almost constant value.

Quantitative discussion on Laurdan fluorescence quenching by QCT

We shall consider the two excited state reaction theory according to which the fluorophore molecule can decay from the excited state (state 1) or from a more relaxed state (state 2). The radiative decay rates from the two states are k_1 and k_2, while the nonradiative decay rates are k_1^{nonrad} and k_2^{nonrad} (Fig. 10).

In this theory (Lakowicz 2006; Tomin et al. 2007) the ratio between the emission intensity from the relaxed state (A_{Red}) and the emission from the non-relaxed state (A_{Blue}) is given by:

$$\frac{A_{\text{Red}}}{A_{\text{Blue}}} = \frac{k_2}{k_1} \cdot \frac{K_{[t^\circ\text{C}]}}{k_2 + k_2^{\text{nonrad}}} \quad (2)$$

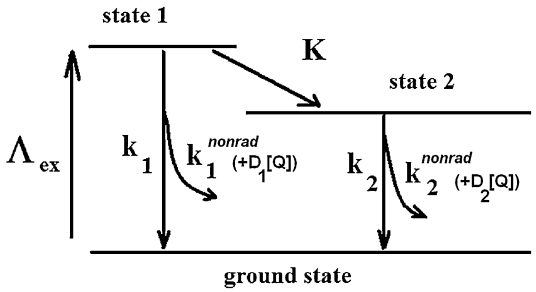

Fig. 10 The representation of the two excited state reaction theory; Λ_{ex} represents the excitation rate for the fluorophore molecule

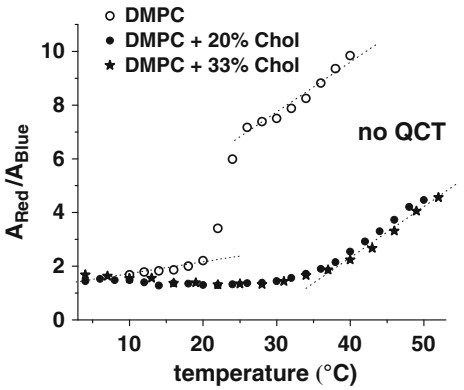

Fig. 11 Emission intensity area ratio dependence on temperature for pure DMPC and DMPC with Chol containing membranes

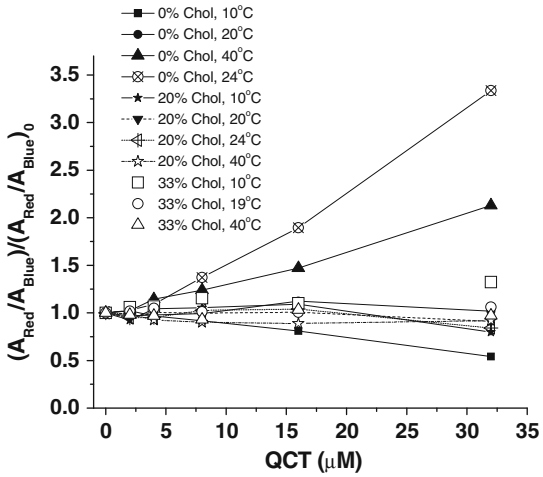

Fig. 12 Normalized fluorescence intensity ratio for the two emitting bands plotted against QCT concentration

where $K_{[t°C]}$ is the relaxation rate at temperature t°C from state 1 to state 2. The fluorescent intensity from each excited state, namely A_{Red} and A_{Blue}, is calculated by multiplying the fit parameters I_{Blue} and I_{Red} with their corresponding bandwidths σ_{Blue} and σ_{Red}. This equation relates the ratio of the two bands emission intensities to the relaxation rate $K_{[t°C]}$ between the two emissive states.

It is obvious from Fig. 11 that the relaxation rate $K_{[t°C]}$ between the two excited states is strongly dependent on the membrane phase, which directly depends on temperature. Its value is small when the pure DMPC membrane is in the gel phase below the main transition temperature and becomes suddenly large when the membrane changes the thermodynamic phase. As for Chol containing liposomes, at temperatures below 35–40°C, the relaxation rate value is quite small and increases with temperature, indicating a fluidization of the membrane in agreement with the known equilibrium phase diagrams for the system phospholipids + Chol (Marsh 2010; McConnell 2010). We can estimate this relaxation rate in the fluid phase of the pure DMPC lipid membrane assuming that the two radiative decay rates are identical and the relaxed state lifetime $\tau_2 = \frac{1}{k_2+k_2^{nonrad}} = 3$ ns (Bagatolli et al. 1997) obtaining $K_{[t°C]}$ of the order 3×10^9 s^{-1}. This relaxation rate depends on the membrane fluidity and on the driving force toward the relaxed state. This driving force might be understood as the force due to the image dipole at the lipid-water interface acting on the dipole moment of the fluorophore in its emitting state (Ionescu and Ionescu 2011).

When QCT is added, the ratio $\frac{A_{Red}}{A_{Blue}}$ normalized to its value at [0] QCT concentration depends not only on temperature, but also on QCT concentration. We chose to represent the normalized ratio to the value corresponding to [0] QCT concentration in Fig. 12.

The two excited state reaction theory gives for the normalized fluorescence intensity ratio:

$$\left(\frac{A_{Red}}{A_{Blue}}\right)\Big/\left(\frac{A_{Red}}{A_{Blue}}\right)_0 = \frac{K_{[q]}}{K_{[0]}} \cdot \left(1 + \frac{D_2}{k_2 + k_2^{nonrad}} \cdot [q]\right) \quad (3)$$

where $K_{[q]}$ and $K_{[0]}$ are the relaxation rates in the presence of $[q]$ QCT concentration and for [0] QCT concentration, respectively; D_2 represents the dynamic quenching rate for the relaxed state (state 2, Fig. 10); $[q]$ is the QCT concentration. Figure 12 is consistent with the expected dependence of the second factor from the above formula on the phase state of the membrane: for the membrane in the gel or ordered phase, one expects a small dynamic quenching constant (pure DMPC liposomes below the phase transition temperature and Chol containing membranes), and for the membrane in fluid phase, one expects larger dynamic quenching constant values (positive slope of the dependence in Fig. 12). The smaller slope of the graphical dependence at higher temperature in the fluid phase might be explained as an increase of the nonradiative decay rates of the relaxed state (decreasing of its lifetime).

The ratio $\frac{K_{[q]}}{K_{[0]}}$, which can be related to the fluidity changes induced by QCT in liposomes, might be responsible for the

small negative slope of the normalized fluorescence intensity ratio dependence on QCT concentration.

The normalized total fluorescence intensity dependence on temperature (Fig. 13) might be understood in the frame of the two excited state reaction theory. Neglecting the possible dependence of the excitation rate Λ_{ex} on temperature, we obtained in the limit of small quantum fluorescence efficiency the following expression:

$$\frac{(A_{\mathrm{Blue}}+A_{\mathrm{Red}})}{(A_{\mathrm{Blue}}+A_{\mathrm{Red}})_{t=10^{\circ}\mathrm{C}}}=1+\left(\frac{k_2}{k_2+k_2^{\mathrm{nonrad}}}-\frac{k_1}{k_1+k_1^{\mathrm{nonrad}}}\right)\cdot\frac{K_{[t^{\circ}\mathrm{C}]}-K_{[10^{\circ}\mathrm{C}]}}{k_1} \tag{4}$$

where $K_{[t^{\circ}\mathrm{C}]}$ stands for the relaxation rate at temperature t°C. As we concluded from Fig. 11, $K_{[t^{\circ}\mathrm{C}]}$ increases with temperature; therefore, $K_{[t^{\circ}\mathrm{C}]}-K_{[10^{\circ}\mathrm{C}]}>0$. The decreasing of total fluorescence with increasing temperature is related to the negative value of the term $\left(\frac{k_2}{k_2+k_2^{\mathrm{nonrad}}}-\frac{k_1}{k_1+k_1^{\mathrm{nonrad}}}\right)$. This leads to the logical result:

$$\frac{k_2}{k_2^{\mathrm{nonrad}}}<\frac{k_1}{k_1^{\mathrm{nonrad}}} \tag{5}$$

which means that the nonradiative transitions are favored from the relaxed state (state 2) for all the types of liposomes that we have studied. In fact, the above condition (5) means that the lifetime of the relaxed state is smaller than the lifetime of the nonrelaxed state, in agreement with previous studies $\tau_1 \approx 6$ ns, $\tau_2 \approx 3$ ns (Bagatolli et al. 1997).

The classical Stern-Volmer plot for the total fluorescence emission intensity is presented in Fig. 14, and the data were fitted with the following exponential function, with two free parameters:

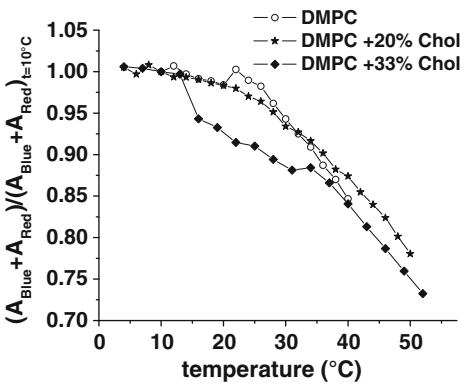

Fig. 13 Fraction areas of the two Gaussian functions (one centered on λ_{blue} and the other centered on λ_{red}) for pure DMPC liposomes and for Chol containing liposomes are plotted versus temperature (the *lines* are drawn to guide the eye)

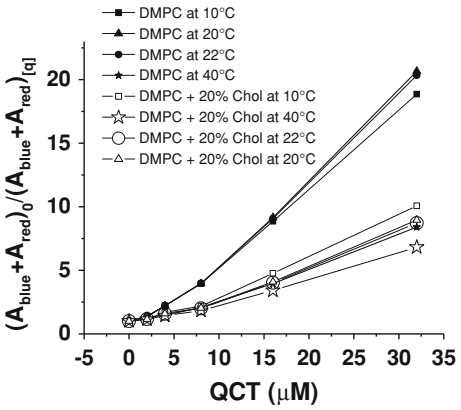

Fig. 14 Stern-Volmer plots: ratio of the total Gaussian area in the absence of QCT on total Gaussian area in the presence of [q] QCT concentrations

$$\frac{(A_{\mathrm{Blue}}+A_{\mathrm{Red}})_0}{(A_{\mathrm{Blue}}+A_{\mathrm{Red}})}=(1+K_{\mathrm{SV}}\times[q])e^{\frac{[q]}{P}} \tag{6}$$

where parameter K_{SV} represents the dynamic quenching rate. The curves that we obtained are upward-curving Stern-Volmer plots, these equations being, in fact, modified Stern-Volmer equations that describe a certain type of apparent static quenching that is usually interpreted in terms of a "sphere of action" within which the probability of quenching is unity (Lakowicz 2006). We have taken into consideration not a "sphere of action," but a "circle of action"; therefore, we correlated the parameter P with the radius of this circle.

Neglecting the possible dependence of the excitation rate Λ_{ex} on QCT concentration, we obtained for $k_1=k_2=k$:

$$\frac{(A_{\mathrm{Blue}}+A_{\mathrm{Red}})_0}{(A_{\mathrm{Blue}}+A_{\mathrm{Red}})}=\left(1+\frac{D_1}{k+k_1^{\mathrm{nonrad}}}\cdot[q]\right)\cdot\frac{N_0}{N}=(1+D_1\cdot\tau_1\cdot[q])\cdot\frac{N_0}{N} \tag{7}$$

when the relaxation rate $K_{[t^{o}C]}$ is small and

$$\frac{(A_{\mathrm{Blue}}+A_{\mathrm{Red}})_0}{(A_{\mathrm{Blue}}+A_{\mathrm{Red}})}=\left(1+\frac{D_2}{k+k_2^{\mathrm{nonrad}}}\cdot[q]\right)\cdot\frac{N_0}{N}=(1+D_2\cdot\tau_2\cdot[q])\cdot\frac{N_0}{N} \tag{8}$$

for large values of the relaxation rate $K_{[t^{\circ}\mathrm{C}]}$. In these equations N_0 is the total number of fluorophore molecules, and N is the number of the fluorophore molecules not statically quenched. Actually, the ratio $\frac{N_0}{N}$ is equal to the term $e^{\frac{[q]}{P}}$ from the exponential fit. Using parameter P from the exponential fit of the modified Stern-Volmer equation, we calculated the radius of the circle of action.

Our pure DMPC samples contained 0.2642 μl of lipids, and the total area of the bilayers was 71.52×10^{-3} m^2 (Nagle and Tristram-Nagle 2000); compared to this volume and to this area, taking into account that quercetin is

hydrophobic and therefore it is probably totally inserted into the membrane core, a 2-μM quercetin concentration in the suspension is equivalent to a 15-mM quercetin concentration in the lipid bilayer volume for pure DMPC liposomes. As for the liposomes containing cholesterol the lipid volumes in 2 ml samples were 0.1796 and 0.1717 μl for 20% respectively 33% cholesterol, while quercetin concentrations related to lipid volume were 19.5 and 18 mM for liposomes containing 20 and 33% cholesterol, respectively. We considered the volume of cholesterol molecules to be $(630 \pm 10) \times 10^{-30}$ m^3 (Greenwood et al. 2006) and the molecular area to be 24×10^{-20} m^2 (Gallova et al. 2010). Although the QCT concentration related to the total lipid volume is quite large, we did not take into consideration a modification of the total lipid surface area induced by quercetin insertion, because due to its flat molecule, we supposed, as previously stated (Tarahovski et al. 2008; Movileanu et al. 2000), that QCT intercalates with its long axis perpendicular on the bilayer surface.

We obtained an average value of (15 ± 3) Å for all types of studied liposomes; this value does not depend on either liposome composition or temperature. We should emphasize that this independence of the quenching circle radius is consistent with Perrin's model (Valeur 2001; Lakowicz 2006).

In Fig. 15 we plotted the dependence of the Stern-Volmer constant as a function of temperature, using the volumetric concentration of the quencher in the lipid, assuming that all QCT molecules were inserted in the lipid phase. From the Stern-Volmer constant in the gel phase of the pure DMPC membrane ~ 50 M^{-1}, using Eq. 7 we obtain a dynamic quenching rate of the nonrelaxed emitting state $D_1 \sim 8 \times 10^9$ M^{-1} s^{-1}. For the high temperature limit of the fluid phase of the pure DMPC membrane, using Eq. 8 we obtain a dynamic quenching rate of the relaxed emitting state $D_2 \sim 5 \times 10^9$ M^{-1} s^{-1}. In the case of Chol containing membranes the Stern-Volmer constant is related

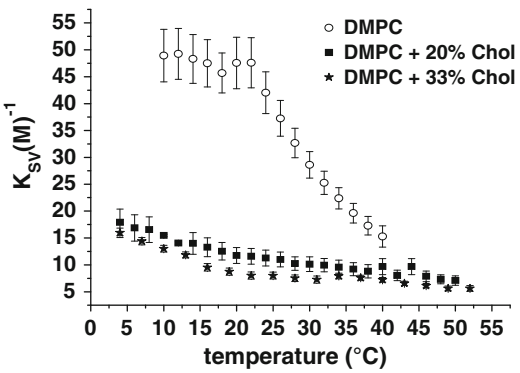

Fig. 15 The dependence of Stern-Volmer constant as a function of temperature for different lipid membrane compositions

to the dynamic quenching rate of the nonrelaxed state (7), and we obtain $D_1^{\mathrm{Chol}} \sim 2.5 \times 10^9$ M^{-1} s^{-1}. The value (D_1^{Chol}) is smaller than D_1 for pure DMPC membranes because the addition of Chol led to an increased degree of lipid order (Parasassi et al. 1994).

Conclusions

Using Laurdan emission spectra we showed that QCT fluidizes the lipid membrane by exponentially decreasing the phase transition temperature for pure DMPC liposomes, as shown by GP calculations. When Chol was part of liposomes, GP gave no information regarding the phase transition temperature. Therefore, we used the decomposition in two Gaussian bands of each Laurdan fluorescence emission spectrum, this kind of decomposition being suggested by the shape of spectra recorded from Chol containing liposomes. It proved that the two Gaussian bands were centered on the two wavelengths corresponding to Laurdan fluorescence emission bands at temperatures below and above phase transition temperatures (440 nm and 490, respectively). Therefore, this approach allowed us to detect the different contribution of the two Laurdan emission bands in a wide range of temperatures.

Our results show that both Laurdan emission states are present to different extents in a wide temperature range for pure DMPC liposomes and as well as for membranes with Chol. At temperatures considerably under phase transition temperature for DMPC, for all the types of liposomes that we studied, the amplitude of Laurdan fluorescence emission from the relaxed state is almost equal to the amplitude of its fluorescence emission from the non-relaxed one. This fact showed that half of Laurdan radiative deexcitation originates from its relaxed excited state, even for Laurdan molecules distributed in the gel phase of the lipids. The simultaneous presence of the two Laurdan excited states in the lipid gel phase is in agreement with previous results (Lucio and De Vequi-Suplicy 2010).

Our experiments also proved that QCT acts as a quencher on Laurdan fluorescence. The quenching is more significant at lower temperatures, meaning that at those temperatures, when lipid packing is tight, water molecules from membrane regions where Laurdan molecules reside are not that numerous and QCT quenches Laurdan fluorescence with a higher efficiency. When the temperature increased above the value corresponding to the phase transition, the lipid membrane was in the liquid-crystal phase and the lipid packing was loose; the amount of water in the vicinity of Laurdan molecules was large, and QCT molecules were probably repelled, therefore leading to a lower efficiency of the quenching.

In order to quantify Laurdan quenching by QCT, we took into consideration the two excited state reaction theory according to which the fluorophore molecule can decay from the excited state (state 1) or from a more relaxed state (state 2), with different radiative and nonradiative decay rates respectively from the two states. We showed that the relaxation rate between the two excited states is strongly dependent on the membrane phase, which also depends directly on the temperature. Its value is small when the pure DMPC membrane is in the gel phase and becomes suddenly large when the membrane changes the thermodynamic phase. As for Chol containing liposomes, at temperatures below 35–40°C, the relaxation rate value is quite small and increases with temperature, indicating a fluidization of the membrane in agreement with the known equilibrium phase diagrams for the system phospholipids + Chol (Marsh 2010; McConnell 2010). We estimated this relaxation rate in the fluid phase of the pure DMPC lipid membrane to be of the order 3×10^9 s^{-1}, assuming that the two radiative decay rates are identical. This relaxation rate depends on the membrane fluidity and on the driving force towards the relaxed state. This driving force might be understood as the force due to the image dipole at lipid-water interface acting on the dipole moment of the fluorophore in its emitting state (Ionescu and Ionescu 2011). We fitted the classical Stern-Volmer plot with an exponential function with two free parameters that represented the dynamic quenching rate and the radius of the quenching circle of action. For the second parameter, characterizing the geometry of quenching, we obtained an average value of (15 ± 3) Å for all types of studied liposomes that did not depend either on liposomes composition or on temperature.

From the Stern-Volmer constant in the gel phase of the pure DMPC membrane ~ 50 M^{-1}, we obtained a dynamic quenching rate of the nonrelaxed emitting state approximately equal to 8×10^9 M^{-1} s^{-1}, while for the high temperature limit of the fluid phase of the pure DMPC membrane, our calculations led to a dynamic quenching rate of the relaxed emitting state equal to $\sim 5 \times 10^9$ M^{-1} s^{-1}. In the case of Chol containing membranes the Stern-Volmer constant is related to the dynamic quenching rate of the nonrelaxed state and we obtained a value of $\sim 2.5 \times 10^9$ M^{-1} s^{-1}. The value proved to be smaller than the corresponding value for pure DMPC membranes because the addition of Chol led to an increased degree of lipid order.

Therefore, we can conclude that our results proved that the relaxed state favors the nonradiative transitions of the fluorophore for all types of liposomes that we studied. Our work shows that when we want to extract the properties of a particular lipid membrane system (phospholipid + Chol + QCT) using the fluorescence spectra of a standard fluorophore (Laurdan), we need to take into account the presence of the fluorescence quenching. In our case QCT proved to be a strong Laurdan fluorescence quencher.

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References

Bagatolli LA, Maggio B, Aguilar F, Sotomayor CP, Fidelio GD (1997) Laurdan properties in glycosphingolipid-phospholipid mixtures: a comparative fluorescence and calorimetric study. Biochim Biophys Acta 1325:80–90

Baran I, Ganea C, Ursu I, Baran V, Calinescu O, Iftime A, Ungureanu R, Tofolean IT (2011) Fluorescence properties of quercetin in human leukemia Jurkat T-cells. Rom J Phys 56:388–398

Chapman CF, Liu Y, Sonek GJ, Tromberg BJ (1995) The use of exogenous fluorescent probes for temperature measurements in single living cells. Photochem Photobiol 62:416–425

De Vequi-Suplicy CC, Benatti CR, Lamy MT (2006) Laurdan in fluid bilayers: position and structural sensitivity. J Fluoresc 16:431–439

Drăguşin M, Tugulea L, Ganea C (2010) The effects of the natural antioxidant quercetin and anions of the Hofmeister series on liposomes marked with chlorophyll a. Gen Physiol Biophys 29: 41–49

Gallova J, Uhrikova D, Kucerka N, Teixeira J, Balgavy P (2010) Partial area of Chol in monounsaturated diacylphosphatidylcholine bilayers. Chem Phys Lipids 163:765–770

Greenwood AI, Tristram-Nagle S, Nagle JF (2006) Partial molecular volumes of lipids and Chol. Chem Phys Lipids 143:1–10

Ionescu D, Ionescu RA (2011) Analytical insights on ion behaviour at interfaces. J Electroanal Chem 650:205–208

Ionescu D, Popescu A, Draguşin M, Dima M, Iftime A, Ganea C (2007) Modulation by QCT of the effect of certain Hofmeister anions on artificial lipid bilayers. Rom J Biophys 17:85–95

Kim YH, Lee YJ (2007) TRAIL apoptosis is enhanced by QCT through Akt dephosphorylation. J Cell Biochem 100:998–1009

Lakowicz JR (2006) Principles of fluorescence spectroscopy, 3rd edn. Springer, NY, pp 277–330

Lucio AD, De Vequi-Suplicy CC (2010) Laurdan spectrum decomposition as a tool for the analysis of surface bilayer structure and polarity: a study with DMPG, peptides and Chol. J Fluoresc 20: 473–482

Marsh D (2010) Liquid-ordered phases induced by Chol: a compendium of binary phase diagrams. Biochim Biophys Acta 1798: 688–699

McConnell H (2010) Adventures in physical chemistry. Annu Rev Biophys 39:1–21

Mezzetti A, Protti S, Lapouge C, Cornard JP (2011) Protic equilibria as the key factor of quercetin emission in solution. Relevance to biochemical and analytical studies. Phys Chem Chem Phys 13: 6858–6864

Movileanu L, Neagoe I, Flonta ML (2000) Interaction of the antioxidant flavonoid QCT with planar lipid bilayers. Int J Pharm 205:135–146

Nagle JF, Tristram-Nagle S (2000) Structure of lipid bilayers. Biochim Biophys Acta 1469:159–195

Parasassi T, De Stasio G, Dubaldo A, Gratton E (1990) Phase fluctuation in phospholipid membranes, revealed by Laurdan fluorescence. Biophys J 57:1179–1186

Parasassi T, Stasio G, Ravagnan G, Rusch RM, Gratton E (1991) Quantization of lipids phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence. Biophys J 60: 179–189

Parasassi T, Di Stefano M, Loiero M, Ravagnan G, Gratton E (1994) Chol modifies water concentration and dynamics in phospholipid bilayers: a fluorescence study using Laurdan probe. Biophys J 66:763–768

Psahoulia FH, Moumtzi S, Roberts ML, Sasazuki T, Shirasawa S, Pintzas A (2007a) QCT mediates preferential degradation of oncogenic Ras and causes autophagy in Ha-RAS-transformed human colon cells. Carcinog 28:1021–1031

Psahoulia FH, Drosopoulos KG, Doubravska L, Andera L, Pintzas A (2007b) QCT enhances TRAIL-mediated apoptosis in colon cancer cells by inducing the accumulation of death receptors in lipid rafts. Mol Cancer Ther 6:2591–2599

Saija A, Scalese M, Lanza M, Marzullo D, Bonina F, Castelli F (1995) Flavonoids as antioxidant agents: importance of their interaction with biomembranes. Free Radic Biol Med 19:481–486

Tarahovski YS, Muzafarov EN, Kim YA (2008) Rafts making and rafts breaking: how plant flavonoids may control membrane heterogeneity. Mol Cell Biochem 314:64–71

Thangasamy T, Sittadjody S, Lanza-Jacoby S, Wachsberger PR, Limesand KH, Burd R (2007) QCT selectively inhibits bioreduction and enhances apoptosis in melanoma cells that overexpress tyrosinase. Nutr Cancer 59:258–268

Tomin VI, Oncul S, Smolarczyk G, Demchenko AP (2007) Dynamic quenching as a simple test for the mechanism of excited-state reaction. Chem Phys 342:126–134

Valeur B (2001) Molecular fluorescence: principles and applications. Wiley-VCH Verlag, Weinheim