The effects of the natural antioxidant quercetin and anions of the Hofmeister series on liposomes marked with chlorophyll a

Magdalena Drăgușin¹, Laura Țugulea² and Constanța Ganea¹

¹ Department of Biophysics, University of Medicine and Pharmacy Carol Davila, 8 Eroii Sanitari Blvd., 050474 Bucharest, Romania; E-mail: magdadragusin@yahoo.com
² University of Bucharest, Faculty of Physics, 405 Atomiștilor Str., 077125 Măgurele, Romania

Abstract. The unilamellar liposomes of dimyristoylphosphatidylcholine (DMPC), marked with chlorophyll a (Chla), have been chosen as suitable models in studies aimed at determining the effects of natural antioxidant quercetin (QCT) and Hofmeister series anions on lipid bilayers. The variations of steady state fluorescence emission intensity of Chla have been recorded and the values of Chla fluorescence anisotropy, under constant temperature and viscosity, at pH = 7.3–7.4 have been measured. Two types of experiments have been performed. In the first type of experiment, the concentration of anions was maintained constant and the concentration of QCT was varied (from 0 to 100 μmol/l). In the second type of experiment, the concentration of QCT was constant (30 μmol/l) and the concentration of anions was varied (from 0 to 152 mmol/l). The quenching of Chla fluorescence by QCT pleads in favor of QCT insertion at interface water-lipid, in the vicinity of the polar heads of lipids from liposomal bilayer at physiological pH and temperature. Fluorescence anisotropy of Chla in liposomes brought more evidences for QCT localizations at lipid/water interface. Chla is sensing a more rigid microenvironment when QCT is added to the lipid bilayer and specific effects of the Hofmeister series anions.

Key words: Quercetin — Hofmeister anions — DMPC liposomes — Chlorophyll a — Spectrofluorimetry

Abbreviations: Chla, chlorophyll a; DMPC, dimyristoylphosphatidylcholine; DMSO, dimethyl sulfoxide; I_{max}, the relative intensity emission maximum; PBS, phosphate buffer solution; ROS, reactive oxygen species; SUVs, small unilamellar vesicles; QCT, quercetin.

Introduction

Multiple biological events that occur at the cellular membrane and interactions of membrane components with the cytoskeleton could be severely affected when damages induced by reactive oxygen species (ROS) occur (Oteiza et al. 2005). It is known that the increased release of free radicals and ROS affects the membrane fluidity, the cellular Ca^{2+} homeostasis and induces lipid peroxidation leading finally to the cell death (Hollán et al. 1996).

Flavonoids like quercetin (QCT) provide antioxidant properties, so that, at the level of cell membrane, they have the capacity to prevent the lipid peroxidation induced by free radicals. QCT (3,3',4',5,7-pentahydroxyflavone) is a natural flavonol, found in many ingredients of the human diet, consumed at highest levels due to its presence in tea, onions, apples (Hertog et al. 1996; Pawlikowska-Pawlega et al. 2007).

The antioxidant properties are determined by chemical structure of QCT, in particular by the presence and location of the hydroxyl (-OH) substitutions and the catechol-type B-ring (Wang et al. 2006). Various authors indicated that QCT, which is characterized by a hydroxylation pattern of 3, 5, 7, 3', and 4' and a catechol B-ring, possesses all the structural elements characteristic of an antioxidant and by exerting its antioxidant activity, QCT may be converted into reactive products (Metodiewa et al. 1999; Rietjens et al. 2005; Harwood et al. 2007).

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Other important factors that contribute to the antioxidant capacity of QCT are the orientation in the membrane lipid bilayer, the degree of incorporation, the uniformity of dis-
bution (Saija et al. 1995; Arora et al. 2000). The pH of the media influences the QCT location within the membrane and its resulting restrictions on the fluidity of membrane components may sterically block the diffusion of free radicals, thus decreasing the kinetics of free radical reactions (Arora et al. 2000; Barros et al. 2001; Ilie et al. 2009 a,b). The embedding of QCT in biological and in model membranes depends, also, on the pH of the media. At acidic pH, QCT is deeply embedded in planar lipid bilayers, while at physiological pH it interacts with the polar head groups at the water-lipid interface (Movileanu et al. 2000; Pawlikowska-Pawlega et al. 2003).

The incorporation is affected by electrostatic interactions, the formation of hydrogen bonds with the polar groups of phospholipids, hydrophobic interactions with fatty acyl chains, and by the molecular geometry of phospholipids (Rego et al. 1995). More general, it is known that the antioxidant efficiency of all flavonoids increases in direct order to their flexibility to conformational changes and in an inverse order to their ability to establish hydrogen bonds. The hydrophobicity of flavonoids and hence their ability to interact with biological membranes is one of the main determinants of their pharmacological activity. So, it was experimentally demonstrated that an inverse correlation between the number of hydroxyl groups and flavonoid hydrophobicity does exist (Rego et al. 1995; Arora et al. 2000; van Dijk et al. 2000; Scheidt et al. 2004). This rule is also applicable for prediction of flavonoids localization in the lipid bilayer as it was demonstrated by NMR spectroscopy (Scheidt et al. 2004). The location of flavonoid molecules in the hydrophobic region or in the polar membrane interface depends on hydrogen bonding of the hydroxyl groups. Molecules of QCT could be comparable with cholesterol, regarding the localization and the influence on the bilayer, increasing the viscosity of lipid bilayer (Tsuchiya et al. 2002; Tarahovsky et al. 2008).

Lyotropic ions or anions of the Hofmeister series induce changes of lipid phase behavior or surface potential and dipolar moment alterations in lipid membranes and liposomes (Sanderson et al. 1991; Clarke et al. 1997, 1999; Koyanova et al. 1997). The Hofmeister series are usually given in terms of the polar moment alteration (Saija et al. 1995; Arora et al. 2000). Liposomes preparation, was purchased from Sigma Aldrich (Germany).

All solutions were prepared using analytical grade reagents and purified water with conductivity ≤0.1 µS·cm⁻¹ from a Millipore Milli-Q system (Billerica, Massachusetts, USA).

Liposomes preparation

Liposomes were prepared according to the thin-film hydration method (R.R.C. New 1990), with little modifications. The lipid DMPC was dissolved in chloroform together with Chla.
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In steady state were done and fluorescence polarization measurements were performed at 36.7°C (the physiological temperature of human body) with accuracy of ±0.3 K ensured the constant temperature (Julabo Labortechnik GmbH 77960 Seelbach/Germany). All the operations have been done and the records were used to calculate the average. The further processing of the data was based on the average values (Figs. 2–6 and Table 1).

In the first type experiment, the concentration of anions was maintained constant (16 and 52 mmol/l, respectively) and the concentration of QCT was varied (from 0 to 100 μmol/l). The control in type I experiments was represented by Chla liposomes in the presence of QCT (from 0 to 100 μmol/l) and without anions. Aliquots of QCT stock DMSO solution have been added in the cuvette solution/suspension to obtain a concentration range of 0–100 μmol/l for every spectral measurement. The DMSO concentration (v/v) in the cuvette did not exceed 0.5%.

In the second type experiment, the concentration of QCT was constant (30 μmol/l) and the concentration of anions was varied (from 0 to 152 mmol/l). The control in type II experiments was represented by Chla liposomes in the presence of anions (16 or 52 mmol/l) or anion concentration, in the presence of fixed QCT concentration (30 μmol/l) and without QCT. Aliquots of anions stock solutions have been added in the cuvette suspension/solution to achieve the desired concentrations.

Results

Steady state fluorescence experiments

Chla fluorescence emission in liposomes suspension was measured in the presence of QCT and anions. The maximum at 430 nm in the Soret band was used as excitation wavelength for all emission spectra. Chla was used as a fluorescent probe and molecular sensor in all the fluorescence experiments. Always control spectra have been recorded, using liposomes with Chla.

As mentioned previously, two types of experiments have been done, by varying either the concentration of QCT added to liposome suspensions, in the presence of anions (16 or 52 mmol/l) or anion concentration, in the presence of fixed QCT concentration (30 μmol/l). In both types of experiments, no wavelength shifts in the
emission spectra have been detected within instrumental resolution limit.

Type I experiments

The relative intensity emission maximum (I_{max}) at 678 nm was monitored and in Fig. 1 the influence of QCT is presented. It is known that Chla is located in the lipid bilayer with the macrocycle at the interface with the water phase, in the vicinity of the lipid polar heads and the phytol in the hydrophobic lipid chains region (Ţugulea et al. 1996). The relative intensity of Chla is decreasing in the presence of QCT, thus QCT is acting as a quencher for Chla fluorescence in liposomes.

The same type of behavior of the relative emission intensity was observed when QCT was added in the presence of all the anions: SO_{4}^{2−}, Cl^{−}, NO_{3}^{−} and ClO_{4}^{−}.

Thus, QCT acts like a quencher of the fluorescence of Chla, without and in the presence of all anions species, and the quenching respects the Stern-Volmer equation (Lakowicz 1983):

$$\frac{\Phi_0}{\Phi} = \frac{I_0}{I} = 1 + K_q \tau_0 [Q] = 1 + K_{SV}[Q]$$

where: $I_0$ and $I$ are the steady-state fluorescence intensities in the absence and presence of quencher, respectively; $K_{SV} = K_q \tau_0$ is the Stern-Volmer constant; $\tau_0$ is the fluorescence lifetime in the absence of the Q; $[Q]$ is the quencher concentration; $K_q$ is the bimolecular quenching constant.

Fig. 2 presents the Stern-Volmer plot for liposomes with Chla, when QCT was added in the presence of the anion (ClO_{4}^{−}), the control being represented by liposomes with Chla, without anion. The data in the insertion of Fig. 2 are fitted well with exponential curve.

A linear Stern-Volmer plot is generally indicative of a single class of fluorophores, all equally accessible to quencher. If two fluorophore populations are present, and one is not accessible to the quencher, then Stern Volmer plots deviate from linearity toward $x$ axis. Much more, a linear Stern-Volmer plot results either in the case of dynamic or static quenching (Lakowicz 1983).

Type II experiments

Fig. 3 presents the variation of relative intensity of Chla fluorescence in type II experiments: in the presence of 30 µmol/l QCT and varied anion concentrations. Some common features for all anions, excluding ClO_{4}^{−}, are notable: Chla fluorescence presents a slight decrease in the presence of QCT. The variation of the relative intensity of Chla in the presence of 30 µmol/l QCT and the three anions: SO_{4}^{2−}, Cl^{−}, NO_{3}^{−}, is fitted by a decreasing exponential curve. In the absence of QCT (control experiment), the presence of varied concentrations of anions has a very small influence on Chla fluorescence.

Fluorescence anisotropy

All fluorescence anisotropy experiments have been done at temperature of 36.7°C, therefore above the transition temperature of DMPC (phospholipid used in all liposome preparations). The emission anisotropy values for Chla in liposomes were recorded using the following wavelengths: $\lambda_{em} = 678$ nm and $\lambda_{exc} = 430$ nm. For each measurement the correction for G factor (the ratio of the sensitivities of the detection system for vertically and horizontally polar-
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(45) was applied. Control experiments for monitoring the possible dilution effect have been done. The addition of buffer solution in volumes corresponding to those of anions aliquots resulted in non significant effects on Chla emission anisotropy and therefore no dilution corrections were necessary.

Fig. 4 presents the anisotropy plot of liposomes with Chla in the presence of the most chaotropic anion, ClO$_4^-$ and varying concentrations of QCT (type I experiment). At the same concentration of QCT, the presence of all anions resulted in lower values for the anisotropy emission. ClO$_4^-$ was chosen because his effect was more evident in this respect.

The fluorescence anisotropy plots in type II experiments are presented in Fig. 5. The presence of anions and their concentrations does not affect at all the anisotropy emission value of Chla, which is rather low. The presence of 30 μmol/l of QCT leads to increasing values for emission anisotropy, no matter the anion nature.

**Figure 3.** Fluorescence emission intensity of Chla in liposomes in the presence of QCT (30 μmol/l) and different concentrations of anions: Na$_2$SO$_4$ (A), NaCl (B), NaNO$_3$ (C), NaClO$_4$ (D) ($\lambda_{exc} = 430$ nm, $\lambda_{em} = 678$ nm).

**Figure 4.** Anisotropy plot of liposomes with Chla in the presence of NaClO$_4$ and different concentrations of QCT.
In all steady-state fluorescence experiments, QCT acts as a quencher for Chla fluorescence. In type I experiments, a quenching obeying the Stern-Volmer equation was obtained in the presence of all anions. The Stern-Volmer plot is linear, indicating that Chla is accessible to the quencher QCT. From the slope of Stern-Volmer plot, $K_{SV}$ was calculated for all samples. Considering a value of 5 ns (Ilík et al. 2003) for the fluorescence lifetime of Chla in the absence of the quencher QCT, the values of bimolecular quenching constants have been calculated. The obtained values for $K_{SV}$ and $K_q$ are presented in Tab. 1.

All $K_q$ values are within the range of $10^{12} \text{M}^{-1} \text{s}^{-1}$ indicating that the quenching process cannot be considered as one

**Table 1.** Bimolecular quenching constants for liposomes with Chla | Anion | $c_A$ (mmol/l) | $K_{SV}$ | $K_q$ ($\times 10^{12} \text{M}^{-1} \text{s}^{-1}$) |
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<tr>
<td>control</td>
<td>0</td>
<td>1.125 ± 0.000441</td>
<td>2.25</td>
</tr>
<tr>
<td>Na$_2$SO$_4$</td>
<td>16</td>
<td>1.076 ± 0.000337</td>
<td>2.16</td>
</tr>
<tr>
<td></td>
<td>52</td>
<td>1.096 ± 0.00043</td>
<td>2.19</td>
</tr>
<tr>
<td>NaCl</td>
<td>16</td>
<td>1.066 ± 0.000335</td>
<td>2.13</td>
</tr>
<tr>
<td></td>
<td>52</td>
<td>0.97 ± 0.000279</td>
<td>1.94</td>
</tr>
<tr>
<td>NaNO$_3$</td>
<td>16</td>
<td>1.011 ± 0.000211</td>
<td>2.02</td>
</tr>
<tr>
<td></td>
<td>52</td>
<td>0.942 ± 0.00036</td>
<td>1.88</td>
</tr>
<tr>
<td>NaClO$_4$</td>
<td>16</td>
<td>1.039 ± 0.000227</td>
<td>2.07</td>
</tr>
<tr>
<td></td>
<td>52</td>
<td>0.886 ± 0.000125</td>
<td>1.77</td>
</tr>
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$c_A$, concentration of anion; control, Chla liposomes + QCT (0–100 μmol/l).

**Discussion**

In all steady-state fluorescence experiments, QCT acts as a quencher for Chla fluorescence.

In type I experiments, a quenching obeying the Stern-Volmer equation was obtained in the presence of all anions. The Stern-Volmer plot is linear, indicating that Chla is accessible to the quencher QCT. From the slope of Stern-Volmer plot, $K_{SV}$ was calculated for all samples. Considering a value of 5 ns (Ilík et al. 2003) for the fluorescence lifetime of Chla in the absence of the quencher QCT, the values of bimolecular quenching constants have been calculated. The obtained values for $K_{SV}$ and $K_q$ are presented in Tab. 1.

All $K_q$ values are within the range of $10^{12} \text{M}^{-1} \text{s}^{-1}$ indicating that the quenching process cannot be considered as one
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limited by diffusion. A dynamic quenching (collisional process) is characterized by bimolecular quenching constants in the range of \(10^{-10} \text{M}^{-1} \text{s}^{-1}\). Therefore, a static process could be considered as responsible for Chla quenching by QCT and in some cases a complex formation in the ground state could be responsible for the quenching process.

In some cases, evidence for the formation of a complex can be obtained (e.g. changes in the absorption spectrum upon complexation), but in the absence of such evidence, the interaction is likely to be non-specific and the model of an effective sphere of quenching is more appropriate (Valeur 2001). Neither changes of the absorption spectra or emission spectra (except variation of the relative intensity) have been observed on liposomes with Chla after adding QCT. An effective sphere of quenching of Chla fluorescence could be assumed and the effect of the Hofmeister series anions could be explained as regarding this sphere.

The calculated \(K_q\) are presented in the Fig. 6, as a plot. A line, representing the regression fit in the case of Cl\(^-\) anion was represented in order to show the distribution of values as regarding the influence of chaotropic and kosmotropic anions on the process of Chla quenching by QCT.

SO\(_4^{2-}\), kosmotropic anion, known as having a "salting-out effect" and distributed in the surrounding water, presents the highest value for \(K_q\), very close to the \(K_q\) value in the absence of anions. The lowest value for \(K_q\) was obtained in the presence of ClO\(_4^-\), anion belonging to the chaotropic category. These anions show the tendency to insert themselves into the hydrophobic part of the membrane ("salting-in effect").

As already proved by other investigations, at neutral pH (Movileanu et al. 2000; Pawlikowska-Pawlega et al. 2003), QCT is inserted in the lipid bilayer. The flavonoid molecule localizes in the hydrophobic region or in the polar region of the membrane interface depending on hydrogen bonding of the hydroxyl groups (Tarahovsky et al. 2008). This incorporation is affected by electrostatic interactions, the formation of hydrogen bonds with the polar groups of phospholipids, hydrophobic interactions with fatty acyl chains, and by the molecular geometry of phospholipids (Arora et al. 2000).

A good explanation for the \(K_q\) values ordering in respect with Hofmeister series anions (SO\(_4^{2-}\) > Cl\(^-\) > NO\(_3^-\) > ClO\(_4^-\) ) could be done considering also the positioning of Chla as regarding the lipid bilayer and QCT inserting into it. The fluorescence properties of Chla are mostly related to its porphyrin macrocycle which is positioned at the interface with surrounding water and in the vicinity of the lipid polar heads (Tugulea et al. 1996). Assuming that the sphere of quenching of Chla is surrounding mainly the Chla macrocycle, the positioning of the "quenching sphere" should be at the interface lipid bilayer-water. The anions could influence the incorporation of QCT by different type of interactions and the different values for \(K_q\) could be explained in correlation with the location of the anions. The kosmotropic anion SO\(_4^{2-}\), distributed in the surrounding water, will have an influence on QCT incorporation mainly by direct electrostatic interaction, and therefore on the effective "quenching sphere". The chaotropic anions, having the tendency to enter in the hydrophobic core of the membrane, will influence the incorporation of QCT, probably affecting the molecular geometry of phospholipids, resulting in an indirect influence of the positioning of the "quenching sphere".

In type II experiments, ClO\(_4^-\), the most effective among investigated chaotropic anions, induced peculiar effects on Chla fluorescence. In the control experiment (liposomes with Chla) and in the presence of QCT, the increase of anion concentration resulted in a slight increase of the relative intensity of Chla fluorescence. The slight increase of Chla fluorescence induced by higher concentrations of chaotropic ion ClO\(_4^-\), in the presence or absence of QCT, could be explained again by the effect of this anion on the molecular geometry of phospholipids. It was shown (Clarke et al. 1999) that in the presence of this anion, having the tendency to enter in the hydrophobic core of the membrane, the increase of dipole moment of lipid is the highest. Chla fluorescence is 'sensing' the lipid molecular geometry change induced by the alteration of the dipole moment due to increasing concentrations of ClO\(_4^-\) anion. The behavior is similar when QCT is present (inserted), the only difference being lower values of the emission relative intensity due to QCT quenching effect.

The fluorescence anisotropy results are supporting the fluorescence emission results.

Chla incorporated in the liposomal membrane exhibited a low degree of emission anisotropy, the values varying between 0.01 and 0.02. This fact suggests that the fluorophore (Chla macrocycle) is localized in a non-rigid microenvironment. As already mentioned, Chla macrocycle is preferen-
tially localized at the interface lipid/water, in the vicinity of lipid polar heads and in close contact with water.

The addition of QCT in both types of experiments resulted always in an increase of the emission anisotropy values. This means that the presence of QCT is providing a more rigid microenvironment for Chla, either by inserting in the vicinity of Chla (“quenching sphere”) or by influencing the microviscosity of the surrounding phospholipids region (polar heads). The anisotropy increase presents a linear dependence on QCT concentration.

At the same concentration of QCT (in type I experiments), the presence of the anions resulted in lower values for the anisotropy emission. Most evident was in the case of $\text{NO}_3^-$ and $\text{ClO}_4^-$, chaotropic anions. A possible explanation is the change in the molecular geometry and order of the phospholipids induced by lipid dipole moment alteration due to the interaction with these chaotropic anions. The new molecular geometry influences the QCT localization in the lipid bilayer and interaction with Chla.

In the case of type II experiments (constant QCT concentration), Chla is sensing a more rigid environment when QCT is added to the lipid bilayer in the presence of all anions.

A linear increase of emission anisotropy upon increasing anion concentration was observed in the case of two anions: $\text{SO}_4^-$ and $\text{Cl}^-$, stronger increase in the case of $\text{SO}_4^-$ anion. This anion is kosmotrop and therefore the anion could be effective mostly in a region close to water (solvent). Chla, localized at the interface lipid/water in the lipid bilayer, is sensing the microviscosity change related to the presence of increasing concentrations of anions only when QCT is present. These results offer more arguments for the insertion of the QCT at the interface lipid/water in the lipid bilayer.

Both fluorescence and emission anisotropy of Chla in liposomes have been done at physiological pH and temperature. The results brought evidences for the insertion of QCT at lipid/water interface, in the vicinity of the polar heads of lipids from liposomal bilayer, which is in agreement with previous studies on BLM (Movileanu et al. 2000). The specific influence of the Hofmeister series anions on QCT localization in the lipid bilayer was proved and explained by both methods. The insertion of QCT in the lipid bilayer, at physiological pH and temperature, has consequences on the microviscosity of lipid moiety in the bilayer. Chla is sensing a more rigid environment when QCT is added to the lipid bilayer and this could be explained by considering a role of QCT similar to cholesterol, as other studies suggested (Tsuchiya et al. 2002; Tarahovsky et al. 2008). Another possible good explanation could be that the addition of QCT to the Chla liposomes is inducing the formation of “lipid rafts” (Tarahovsky et al. 2008) in the close vicinity of Chla. The influence of the Hofmeister series anions on QCT effects is depending on the specific interaction of the anions, either directly on QCT insertion in the bilayer or indirectly, by alteration of molecular geometry of phospholipids.

Chla, used as fluorescence marker for liposomes, was successfully exploited as sensor at molecular level for revealing specific influence of QCT and anions from Hofmeister series on liposomal membrane.

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