THE JOURNAL OF PHYSICAL CHEMISTRY B

ARTICLE

pubs.acs.org/JPCB

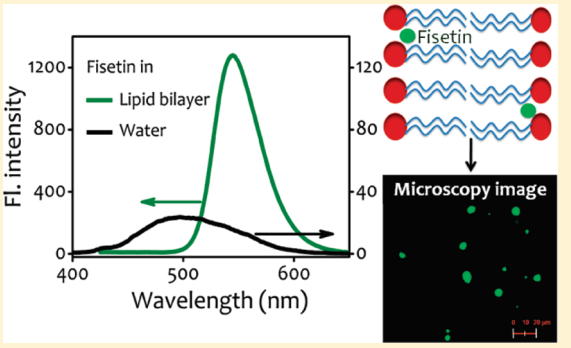
Photophysical Behavior of Fisetin in Dimyristoylphosphatidylcholine Liposome Membrane

Monalisa Mohapatra and Ashok K. Mishra*

Department of Chemistry, Indian Institute of Technology Madras, Chennai 600 036

Supporting Information

ABSTRACT: A detailed photophysical study of the plant flavonoid fisetin in a dimyristoylphosphatidylcholine (DMPC) bilayer membrane has been carried out. Fisetin is found to partition well into the membrane ($K_p = (4.6 \pm 0.5) \times 10^5$ in solid gel phase and $(5.1 \pm 0.5) \times 10^5$ in liquid crystalline phase). A fluorescence quenching study using cetylpyridinium chloride (CPC) as the quencher suggests that fisetin molecules are generally present near the head group region of the lipid bilayer membrane. The temperature dependence of the fluorescence lifetime indicates a local heterogeneity in the distribution of fisetin within the bilayer membrane. The phototautomer form of fisetin, which is the primary emitting species from the lipid membrane, has a large Stoke's shift (175 nm) and fluoresces with an intense green fluorescence, which can make the molecule a good dye for marker and bioimaging applications. Membrane-bound fisetin shows sensitive variations of fluorescence intensity, lifetime, and anisotropy parameters in cholesterol-containing DMPC membranes, in mixed phospholipids, and as a function of temperature. This suggests that fisetin can be an efficient fluorescent molecular probe for sensing lipid bilayer membrane related changes. The location of fisetin in the membrane and the observed cholesterol-induced expulsion of fisetin may possibly have implications in the antioxidant activity of fisetin.



INTRODUCTION

Flavonoids are important polyphenolic compounds under the class of plant secondary metabolites and are also most commonly found in the human diet. These are very significant in the biological systems because of their low toxicity, and from the in vitro experimental studies, it is evident that they can modify allergens, viruses, and carcinogens.[1,2] Therefore, they have potential to be biological "response modifiers" such as antiallergic, antimutagenic, anti-inflammatory, antihemolysis, antimicrobial, anticancer, and anti-AIDS activities.[1−6] In addition, flavonoids are also very important for their antioxidant activity in vitro. Apart from these properties, the photophysical characteristics of flavonoids are very interesting due to their highly sensitive fluorescence behavior that arises from the photoinduced excited-state intramolecular proton-transfer (ESIPT) processes.[6−12] Fisetin is considered one of the best models for the study of ESIPT due to its existence in different prototropic forms depending on the surrounding microenvironment,[13−16] as illustrated in Figure 1.

The absorption and emission spectra of fisetin are strongly solvent-dependent,[13,14] which enables the molecule to be used as a laser dye.[12] This is because of the solvent sensitivity of the ESIPT process in fisetin. The molecular-level interactions of fisetin with a variety of organized systems like micelles, reverse micelles, cyclodextrins, DNA, and proteins have been studied through various fluorescence parameters.[6,14−18] The binding and location of some flavonoids, like 3-hydroxyflavone,[19] 7-hydroxyflavone,[20] and so forth, in lipid bilayer membranes have been studied. With regard to fisetin in a lipid bilayer membrane, however, there is just one report by Sengupta et al.,[15] which shows that in a liposome medium, the predominant fluorescence emission of fisetin is due to its phototautomer form (λ_{ex} = 370 nm and λ_{em} = 545 nm). Although it has been suggested that the probable location of the membrane-bound fisetin is at the bilayer interface,[15] there is no specific experiment to date to establish it conclusively.

The aim of this work is to study the photophysical behavior of fisetin incorporated in liposomes composed of a disaturated phospholipid, dimyristoylphosphatidylcholine (DMPC), in greater detail. The response of fisetin fluorescence to conditions like temperature, lipid composition, and the presence of cholesterol, which are known to alter the bilayer properties, has been studied in depth.

MATERIAL AND METHODS

Materials. Fisetin (Sigma−Aldrich, Bangalore, India) was used without further purification. DMPC was purchased from Sigma Chemical Co. (Bangalore, India) and was used as

Received: December 28, 2010
Revised: July 15, 2011
Published: July 18, 2011

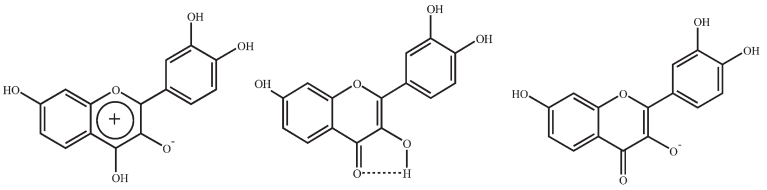


Figure 1. Prototropic forms of fisetin, neutral, phototautomer, and anionic, with their corresponding excitation and emission wavelengths.

such. Cetylpyridinium chloride (CPC) was purchased from SRL (India) and was used as received. All of the solvents used were of spectral grade. Water, distilled twice from alkaline permanganate solution, was used for the experiments.

Liposome Preparation. Small unilamellar vesicles (SUVs) were used for most of the studies. For experiments involving cholesterol incorporation, multilamellar vesicles (MLVs) were used. SUVs were prepared by ethanol injection method.[21] The stock solution of the lipid was prepared in ethanol. The desired amount of ethanolic solution of lipid was injected rapidly into the aqueous solution of fisetin equilibrated for 30 min at 50 °C. The percentage of ethanol in the solution was less than 1% (v/v). For MLVs, the lipid was dissolved in chloroform at the required molar concentration.[21] The solution was evaporated with the help of a rotary evaporator, and the residual solvent was removed by applying vacuum. The lipid film was left under vacuum to ensure complete dryness. Liposome vesicles were prepared by adding an appropriate volume of an aqueous solution of fisetin at neutral pH to the dry lipid film with vigorous vortexing and then warming at 10° above the phase transition temperature for complete hydration. DMPC-cholesterol liposomes were prepared by adding same volume of lipid to different volumes of cholesterol stock in chloroform, such that the molar ratio of cholesterol varied from 0 to 50 mol % of the lipid. For all experiments other than that for the determination of K_p, the [lipid] was fixed at 0.4 mM and that of fisetin at 4 μM. The low concentration of 0.4 mM lipid was used to ensure that the ethanol injection method gave a single phase of the unilamellar vesicle membrane. Given the low solubility of fisetin in water, the low concentration of fisetin used was to make sure that all of the fisetin used was completely soluble in aqueous phase. The low ratio of [fisetin]/[lipid] at 1:100 was used for experiments to minimize the probe-induced changes in the bulk physical properties of the membrane. All experiments were performed with freshly prepared liposome suspensions as well as fisetin solutions.

Fluorescence Measurements. Fluorescence measurements were carried out with a Hitachi F-4500 spectrofluorometer. The emission spectra were recorded with slit widths of 5/5 nm by fixing excitation wavelengths at 370 nm for the phototautomer form of fisetin. The temperature was controlled by circulating water through a jacketed cuvette holder from a refrigerated bath (JULABO, Germany). The steady-state fluorescence anisotropy (r_{ss}) values were obtained by using the expression

$$r_{ss} = \frac{(I_{\parallel} - GI_{\perp})}{(I_{\parallel} + 2GI_{\perp})} \quad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ are fluorescence intensities when the emission polarizer is parallel and perpendicular, respectively, to the direction of polarization of the excitation beam and G is an instrument correction factor.

Fluorescence Lifetime Measurements. The fluorescence lifetime measurements were carried out using a Horiba Jobin Yvon TCSPC lifetime instrument in a time-correlated single-photon counting arrangement. A 370 nm nano-LED was used as the light source. The pulse repetition rate was set to 1 MHz, and the instrumental full width at half-maximum of the 370 nm LED, including the detector response, was ~1.1 ns. The instrument response function was collected using a scatterer (Ludox AS40 colloidal silica). The decay data were analyzed using IBH software. A value of χ^2, $0.99 \leq \chi^2 \leq 1.2$, was considered as a good fit, which was further judged by the symmetrical distribution of the residuals. The average fluorescence lifetime (τ_{avg}) values were obtained by the following equation[22]

$$\tau_{avg} = (\sum_{i=1}^{n} \alpha_i \tau_i^2)/(\sum_{i=1}^{n} \alpha_i \tau_i) \quad (2)$$

where τ_i is the individual lifetime with corresponding amplitude α_i.

Fluorescence Microscopic Imaging. An Olympus IX71 fluorescence microscope attatched with an Andor CCD camera interfaced to a computer and operated by Andor IQ software was used for the imaging of fisetin embedded in DMPC MLVs. Subsequent image processing was performed using ImageJ software. Chroma (71000aV2 set) filters were used with an excitation band-pass at 380 nm and emission from 470 to 550 nm.

RESULTS AND DISCUSSION

Fluorescence Spectral Studies of Fisetin in DMPC Vesicles. The excitation (λ_{em} = 500 nm) and emission (λ_{ex} = 370 nm) spectra of fisetin in neutral water are shown in Figure 2A; the inset shows a red shift in the emission maximum of fisetin from water (500 nm) to liposome (545 nm) media. The excitation (λ_{em} = 545 nm) and emission (λ_{ex} = 370 nm) spectra of fisetin in both solid gel (SG) and liquid crystalline (LC) phases of DMPC liposome are shown in Figure 2B. The figure shows a ~50% drop in the fluorescence intensity of fisetin as the membrane transits from the SG to LC phase. The excitation spectra of fisetin in Figure 2A and B show that the predominant light-absorbing form in the ground state is the neutral form, in aqueous as well as liposome media. However, the predominant light-emitting form is the photoanion (λ_{ex} = 370 nm, λ_{em} = 500 nm) in an aqueous medium and the phototautomer (λ_{ex} = 370 nm, λ_{em} = 545 nm) in a liposome medium. The phototautomer shows almost 50-fold

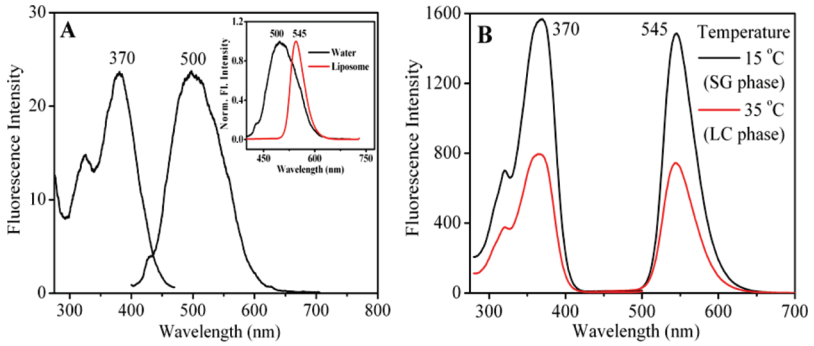


Figure 2. (A) Excitation (λ_{em} = 500 nm) and emission (λ_{ex} = 370 nm) spectra of fisetin in neutral water; the inset shows normalized emission spectra in water and liposome. (B) Excitation (λ_{em} = 545 nm) and emission spectra (λ_{ex} = 370 nm) of fisetin in DMPC liposome at 15 (SG phase) and 35 °C (LC phase); [fisetin] = 4 μM.

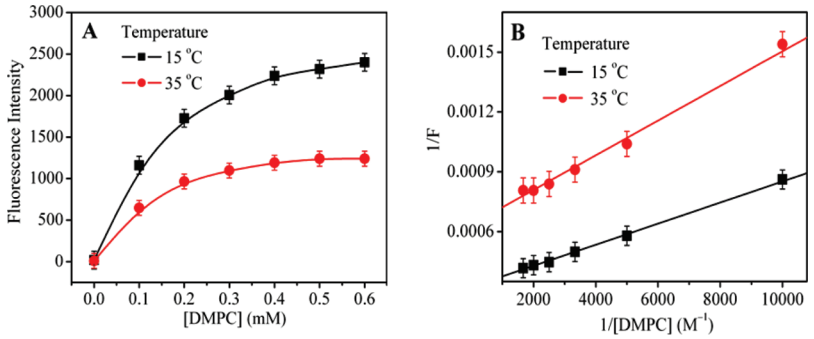


Figure 3. (A) Plots of variations of the phototautomer fluorescence intensity and (B) a double reciprocal plot of the phototautomer fluorescence intensity with respect to DMPC concentration at the SG (15 °C) and LC (35 °C) phases; λ_{ex} = 370 nm, λ_{em} = 545 nm, [fisetin] = 5 μM.

enhancement in the fluorescence intensity as compared to the photoanion in water.

Determination of the Partition Coefficient (K_p). The rather limited literature available in the study of fisetin−liposome interactions does not give the partition coefficient (K_p) value. This value is important due to its obvious implications in the biological and antioxidant activity of fisetin.

Figure 3A shows the variation of the fluorescence intensity of the phototautomer form (λ_{ex} = 370 nm) with DMPC concentration at SG and LC phases. The partition coefficient (K_p) of fisetin is defined as[23]

$$K_p = \frac{(C_L/L)}{(C_W/W)} \tag{3}$$

where C_L is the molar concentration of membrane-bound fisetin, C_w is the molar concentration of free fisetin in aqueous phase, L is the molar concentration of lipid, and W is the molar concentration of water. Because fisetin in a lipid bilayer membrane (phototautomer form, 545 nm) is almost 100 times more fluorescent as compared to that of free fisetin in water (photoanion) at the same wavelength, the fluorescence (F) of fisetin at 545 nm is proportional to the concentration of membrane-bound fisetin ($F \propto C_L$). Because the total fisetin concentration is $C = C_L + C_W$, eq 3 can be arranged to

$$F = \frac{F_0 L}{(W/K_p + L)} \tag{4}$$

where, $F_0 \propto C$, and it is the fluorescence resulting from maximum fisetin incorporation into the membrane. Assuming the molar concentration of water to be 55.6 M, eq 4 may be rewritten as

$$F = \frac{F_0 L}{(55.6/K_p + L)} \tag{5}$$

or

$$\frac{1}{F} = \frac{55.6}{(K_p F_0 L)} + \frac{1}{F_0} \tag{6}$$

Using eq 6 and the slopes from Figure 3B, the calculated K_p values at 15 and 35 °C are nearly the same, that is, $(4.6 \pm 0.5) \times 10^5$ and $(5.1 \pm 0.5) \times 10^5$, respectively, with [fisetin] at 5 μM and [DMPC] at 0.4 mM. These partition coefficient values are reasonably large, implying that about 78% of fisetin is partitioned to the lipid bilayer membrane.

Possible Use of Fisetin Tautomer Fluorescence as a Marker and for Fluorescence Imaging. Fisetin in a lipid bilayer membrane with (i) a large Stoke's shift of ~175 nm (excitation maximum (370 nm) to emission maximum (545 nm)), (ii) a Gaussian spectral profile, (iii) emission in the green region (middle of the visible spectrum), (iv) a reasonably high extinction coefficient (2.6×10^4 M^{-1} cm^{-1}) at the absorption maximum (370 nm), (v) a large K_p value, and (vi) an almost 50 fold enhancement in intensity with respect to water (Figure 2) makes it a suitable molecule for fluorescence imaging studies and to be used as a marker in biological systems. As seen in Figure 4, the fluorescence microscopic

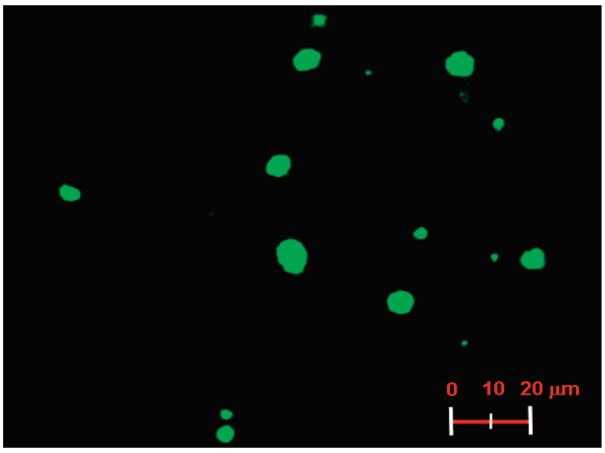


Figure 4. Fluorescence microscopic image of fisetin embedded in DMPC multilamellar vesicles; [fisetin] = 5×10^{-5} M, [DMPC] = 0.6 mM.

image of fisetin molecules embedded in DMPC MLVs permits visualization of the size distribution of MLVs in the range of ~1 to 10 μm.

Apart from the extent of partitioning, the information of possible location of fisetin in the bilayer membrane is very important to understand its microenvironment. Previous fluorescence studies on fisetin in liposome suggest its preferential location at the interfacial region of the bilayer[15] with some heterogeneity in the distribution. However, the exact location of the phototautomer form can be understood from a fluorescence quenching study.

Fluorescence Quenching Study. A fluorescence quenching study has been performed with CPC as the quencher. CPC is a well-known surfactant with a 16-carbon chain and acts as an effective antiplaque, antigingivitis, and antiseptic agent.[24] The binding of CPC with giant unilamellar vesicles made of a phosphatidylcholine (PC) lipid POPC has been studied in detail by Arrigler et al.[25] at [lipid] = 7.3 μM and up to [CPC] = 5.6 μM (molar ratio of [CPC]/[lipid] at 0.76:1), and quantitative partitioning of CPC to the lipid bilayer occurs. It has also been shown that below [CPC]/[lipid] of nearly 2:1, the surfactant−lipid mixed vesicles remain stable in their spherical shape. Because a PC is used for liposome preparation in our work, a similar partition efficiency of CPC to the bilayer membrane is expected at the concentration of lipid (0.4 mM) and CPC (≤0.3 mM) used in this study, which gives a molar ratio of [CPC]/[lipid] at 0.75:1. It is also to be noted that the concentration of CPC used in the study is well below the CMC of CPC (0.98 mM).[26]

With a positive pyridinium moiety, the head group of the surfactant is expected to be positioned near the negatively charged phosphate head group of the phospholipid molecule, thereby quenching the fluorescence originating in the interfacial region. In previous work, while studying the interaction of the fluorescent molecule 1-(4-*N*,*N*-dimethylaminophenylethynyl)pyrene (DMAPEPy) with PC lipid bilayer membranes, it was found that DMAPEPy distributes both in the core and interfacial region of the membrane.[27] The emission from the core location was structured and at shorter wavelengths, and that from the interfacial region was broad and red-shifted. The molecule was almost nonfluorescent in water. When CPC was used as a fluorescent quencher, there was preferential quenching of the broad and structureless fluorescence originating from the interface, and the structured spectrum originating from core remained unquenched. This showed that the quenching action of CPC is confined to the interfacial region. In another study, using mixed fluorophores (pyrene and anthracene derivatives) in SDS micellar medium, it has been concluded that CPC quenches fluorescence from fluorophores at the micelle−water interface, whereas the fluorophores in the micellar core are significantly less quenched.[28]

For further mechanistic insights on the quenching efficiency of CPC, fluorescence quenching experiments have been performed in homogeneous as well as liposome media. It was found that THF as a solvent medium is suitable for such a study because fisetin is known to predominantly emit from its phototautomer form in THF[16,29] and CPC is reasonably soluble in this solvent. Figure 5A show the emission spectra of fisetin in THF at 25 °C with varying concentrations of CPC. The Stern−Volmer plot (F_0/F versus [CPC]) for the quenching of phototautomer fluorescence is given in Figure 5B. The Stern−Volmer quenching constant, K_{sv}, is calculated from the slope of the linear plot based on the following equation[22]

$$\frac{F_0}{F} = 1 + K_{sv}[Q] \tag{7}$$

where F_0 and F are fluorescence intensities in the absence and in the presence of the quencher, respectively; $[Q]$ is the concentration of the quencher.

The phototautomer fluorescence intensity at 545 nm decreases gradually with the addition of CPC (up to 1 mM). The diffusion-controlled quenching ($k_{diffusion}$) as estimated from the viscosity of THF at 25 °C is 1.44×10^{10} M^{-1} s^{-1}. The fluorescence lifetime of the phototautomer form in THF without a quencher (τ_0) was found to be 0.8 ns. Using the values of $k_{diffusion}$ and τ_0, the estimated maximum value of K_{sv} would be 11.52 M^{-1} if the fluorescence quenching were dynamic. However, the linear F_0/F plot gives a K_{sv} value of 6.91×10^2 M^{-1} at 25 °C. The high value of K_{sv} suggests the fluorescence quenching to be static in nature. This conclusion is further substantiated from the τ_0/τ plot in Figure 5B, which shows that there is almost no change in the fluorescence lifetime of the phototautomer form with the addition of CPC; the plot of τ_0/τ against [CPC] is almost parallel to the *x*-axis.[22] The corresponding lifetime decay plots with the addition of CPC in THF are given in the Supporting Information (Figure S1).

Having ascertained the quenching of phototautomer fluorescence in the THF medium to be static in nature, the quenching study was further carried out in a liposome medium. Figure 6A and B shows the emission spectra of fisetin in DMPC unilamellar vesicles at 15 and 35 °C, respectively, with varying concentrations of CPC. The Stern−Volmer plots for the quenching of fisetin fluorescence are given in Figure 6C and D.

As compared to the homogeneous THF medium, more appreciable quenching in the phototautomer fluorescence intensity was observed even at low concentrations (0.3 mM) of CPC in the liposome medium. The quenching plots show good linearity with large K_{sv} values of 3.05×10^4 and 0.93×10^4 M^{-1} at 15 (SG phase) and 35 °C (LC phase), respectively, strongly indicating the occurrence of static quenching. Because it is known that the phototautomer form of fisetin emits only from the membrane-bound fisetin, these large apparent K_{sv} values

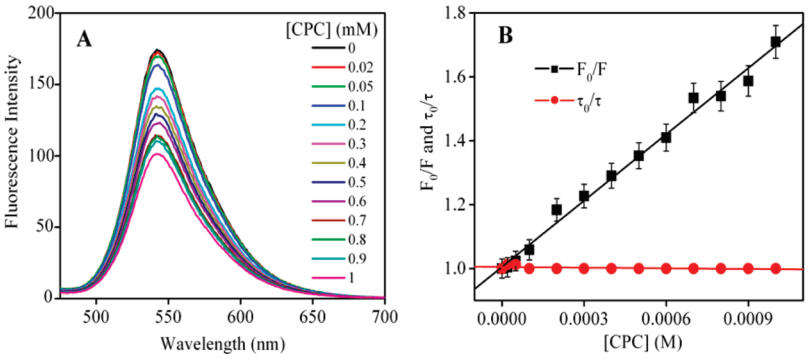


Figure 5. (A) Emission spectra of fisetin (λ_{ex} = 370 nm) in THF at 25 °C, with varying concentrations of CPC; (B) Stern−Volmer plot (F_0/F versus [CPC]; R = 0.995, K_{sv} = 6.91 × 10^2 M^{-1}) and τ_0/τ plot for the quenching of the phototautomer form of fisetin in THF by CPC; [CPC] = 0 to 1 mM, [fisetin] = 4 μM.

imply a locally enhanced concentration of CPC in the membrane phase. In the fluidic LC phase, the area per hydrated head group (∼60 Å^2) is more than that in the compact SG phase (∼45 Å^2) of the membrane.[30,31] Thus, the lesser static quenching in the LC phase as compared to that in the SG phase is possibly due to a longer mean distance between the fisetin molecule and the quencher head group of CPC in the fluidic LC phase as compared to that in the compact SG phase. The static nature of fisetin phototautomer form fluorescence quenching by CPC is better understood from the τ_0/τ plot in Figure 6C and D. The decrease in τ with the concentration of CPC is negligible as compared to the decrease in the fluorescence intensity, and thus, the plot of τ_0/τ against [CPC] is almost parallel to the x-axis[22] in both SG and LC phases. The corresponding lifetime decay plots with the addition of CPC at both phase states of the membrane are given in the Supporting Information (Figures S2A and S2B). The phototautomer form emission originates exclusively from the membrane-bound fisetin, and the CPC head group is positioned near the head group of the phospholipid molecule. Thus, from the effective quenching of the phototautomer fluorescence intensity, it is evident that fisetin molecules are located near the head group region of the lipid bilayer membrane.

The location of fisetin at the head group region of the lipid bilayer membrane can possibly have significant implications in its antioxidant activity. It has been proposed that the antioxidant activity of flavonoids in liposomes is significantly influenced by their location in the membrane and the site of generating free radicals.[32] Depending on the extent of hydrophobicity; antioxidants are known to be located either at the membrane core region or in the interfacial head group region. Some less polar flavonoids are known to partition into the hydrophobic core of model membranes, resulting in modification of the lipid packing order, thereby partially fluidizing the membrane.[33] However, polar flavonoids being located at the interfacial region would not lead to membrane-related changes. In addition, the flavonoids that are known to be located in the polar surface region of the lipid bilayer can easily trap aqueous peroxyl radicals. Therefore, they are more accessible to chain-initiating radicals than the more hydrophobic antioxidants located within the membrane.[34] Fisetin appears to belong to the later group of antioxidants, being located at the head group region.

Fisetin as a Membrane Probe. Fluorescent molecules are often used as membrane probes to study the membrane-related changes by external purturbers like temperature, pressure, cholesterol, ethanol, and surfactants.[9,35−37] An efficient membrane probe includes properties like high affinity toward the lipid bilayer membrane and large changes in one or more of its fluorescence parameters after its incorporation into the liposome medium. Here, we have tried to use fisetin as a membrane probe due to its higher partitioning efficiency and remarkable change in fluorescence parameter from the water to liposome medium, a red shift in the fluorescence maxima of ∼45 nm upon going from water (500 nm) to liposome medium (545 nm) with a significant enhancement of fluorescence intensity. Using fisetin, we have studied (i) the temperature-dependent phase changes in the bilayer, (ii) the effect of cholesterol on membrane properties, and (iii) the phase behavior of a mixed lipid system (DMPC and DPPC).

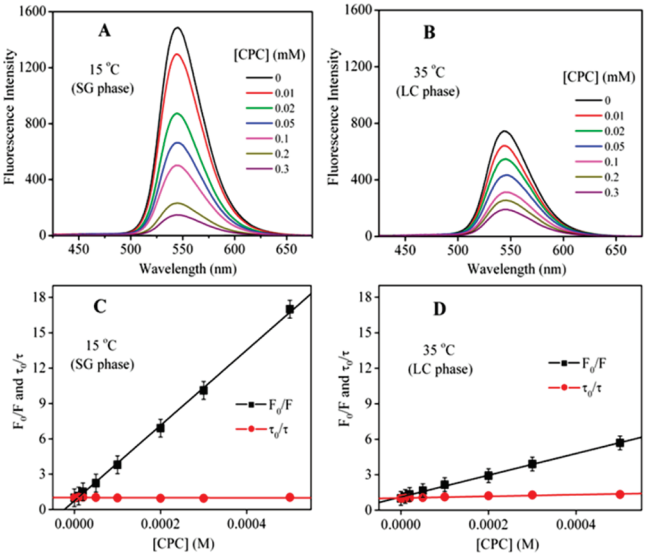


Figure 6. Emission spectra of fisetin (λ_{ex} = 370 nm) in DMPC unilamellar vesicles (0.4 mM) with varying concentrations of CPC at (A) 15 (SG phase) and (B) 35 °C (LC phase); Stern−Volmer plot (F_0/F versus [CPC]) and τ_0/τ plot for the quenching of the phototautomer form in DMPC vesicles by CPC at (C) 15 (R = 0.999 and K_{sv} = 3.05 × 10^4 M^{-1}) and (D) 35 °C (R = 0.997 and K_{sv} = 0.93 × 10^4 M^{-1}); [CPC] = 0−0.3 mM, [DMPC] = 0.4 mM, [fisetin] = 4 μM.

Temperature-Dependent Studies. Phospholipid bilayers are known to change from a highly ordered SG phase to a fluidic LC phase with an increase in temperature.[21] This phase change

dx.doi.org/10.1021/jp1123212 |J. Phys. Chem. B 2011, 115, 9962–9970

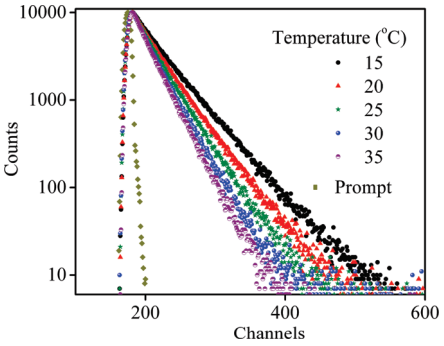


Figure 7. Fluorescence lifetime decay profiles of the phototautomer form of fisetin in DMPC vesicles at different temperatures. The rising point of the response starts at around the 160th channel. Time calibration = 1.122019E^{-10} s/channel, λ_{ex}= 370 nm, λ_{em}= 545 nm, [DMPC] = 0.4 mM, [fisetin] = 4 μM.

Table 1. Fluorescence Lifetime Data of the Phototautomer (λ_{ex}= 370 nm, λ_{em}= 545 nm) in DMPC Vesicles[a]

temp. (°C)	τ_1 (α_1)	τ_2 (α_2)	τ_{avg}	χ^2
15	5.4_4 (77)	2.8_8 (23)	5.0_9	1.10
20	4.4_1 (83)	2.4_4 (17)	4.2_1	1.02
25	3.8_2 (87)	1.8_7 (13)	3.6_9	1.06
30	3.4_0 (85)	1.7_2 (15)	3.2_6	0.99
35	3.0_0 (92)	1.0_2 (8)	2.9_4	1.03

[a] [DMPC] = 0.4 mM; [fisetin] = 4 μM.

in biological membranes affects numerous vital membrane functions. The response of a potential molecular probe to the phase transition in membranes is often taken as a convenient test for its applicability in this type of studies. In this work, the efficiency of fisetin as a probe in the temperature-induced phase transition of membrane is investigated from temperature-dependent lifetime, steady-state fluorescence emission, and steady-state fluorescence anisotropy studies.

Fluorescence lifetime study has been performed to ascertain the change in fluidity of the DMPC membrane with temperature and establish the local heterogeneity in the distribution of phototautomers. With an increase in temperature, the average lifetime (τ_{avg}) values and short (τ_2) as well as long (τ_1) lifetime components are found to decrease (Figure 7, Table 1). The measured drop in average lifetime value is ~42% as the membrane fluidity increases with an increase in temperature from 15 (SG phase) to 35 °C (LC phase), which nearly agrees with the drop in fluorescence intensity of the phototautomer (Figure 2B). This near-correspondence in the changes of fluorescence and lifetime suggests that there is an increase in nonradative decay (k_{nr}) processes upon going from the SG phase to the more fluid LC phase.

The decay profile is also found to be biexponential in nature with good χ^2 values (Table 1), which suggests some local heterogeneity in the distribution of the phototautomer, although its location is established to be mostly at the interfacial region of bilayer. The occurrence of heterogeneity could possibly be due to different modes of interaction of the fisetin hydroxyl groups with the ester linkages at the interfacial region.

The fundamental fluorescence anisotropy (r_0) of the phototautomer form of fisetin measured in glycerol at −10 °C was found to be 0.36. In the homogeneous THF medium, the phototautomer form shows a steady-state fluorescence anisotropy (r_{ss}) value of 0.06 at 25 °C. In the liposome medium, it has the values of 0.27 at 15 °C and 0.22 at 35 °C, which are significantly higher than that in th homogeneous THF medium. The higher r_{ss} value of the phototautomer form of fisetin in the liposome medium than that in THF gives information regarding the restriction in the rotational mobility of the phototautomer form in the liposome medium.

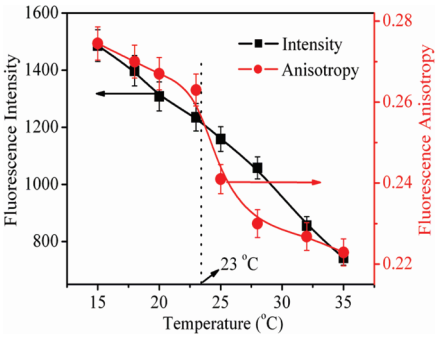


Figure 8. Plots of the variation of the steady-state fluorescence intensity and anisotropy of the phototautomer (λ_{ex}= 370 nm, λ_{em}= 545 nm) with temperature in DMPC vesicles; [DMPC] = 0.4 mM, [fisetin] = 4 μM.

Figure 8 shows the variation in steady-state fluorescence intensity and anisotropy of the phototautomer in DMPC vesicles at a temperature range of 15−35 °C. A sigmoidal decrease in anisotropy is observed with an increase in the membrane fluidity[15,19] amid a maximum change at the phase transition temperature (~23 °C).

Figure 8 shows gradual decrease in the fluorescence intensity of the phototautomer as the membrane transits from the highly ordered SG phase to the fluidic LC phase. It has been observed that the measured drop in fluorescence intensity of the phototautomer is ~50% (Figure 2B), whereas the drop in average lifetime is ~42% (Table 1). Thus, the decrease in the phototautomer intensity can be attributed to the increase in k_{nr} upon going from the SG phase to the more fluid LC phase as a major contributing reason. The considerable change in the phototautomer anisotropy of fisetin with temperature signifies its sensitivity to the phase change in the membrane and its efficiency as a membrane probe. However, the information of phase transition is not clear due to the nonsigmoidal decrease in fluorescence intensity and can be ascribed to the heterogeneity in the distribution of the phototautomer in the DMPC membrane, which has been established from the fluorescence lifetime study.

Sensitivity of Fisetin to the Effect of Cholesterol on DMPC Bilayer. The use of fisetin as a fluorescent molecular probe has also been evaluated by following the changes in the fluorescence of the phototautomer form with increasing incorporation of cholesterol in the lipid bilayer membrane. Cholesterol mostly consists of a planar steroid nucleus, a 3β-hydroxyl group, and an aliphatic side chain.[21] Therefore, being amphipathic in nature, its hydrophobic moiety tends to insert into the hydrocarbon core region of the membrane with the hydroxyl group oriented toward the aqueous surface. This type of insertion of cholesterol into the bilayer membrane results in the alteration of acyl chain order in both the SG as well as LC phase of the bilayer. At the SG phase, the hydrophobic moiety can push apart the tightly packed phospholipids, which results in an increase in the fluidity of the

dx.doi.org/10.1021/jp1123212 |J. Phys. Chem. B 2011, 115, 9962–9970

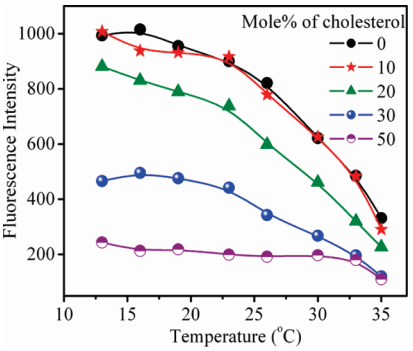


Figure 9. Variation of the fluorescence intensity of fisetin at 545 nm with temperature and varying amounts of cholesterol in DMPC MLVs; λ_{ex} = 370 nm, λ_{em} = 545 nm, [fisetin] = 4 μM, [DMPC] = 0.4 mM, [cholesterol] = 0−50 mol %.

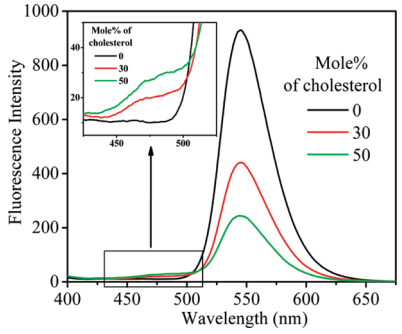


Figure 10. Emission spectra of fisetin at different cholesterol concentrations in DMPC MLVs at 13 °C; λ_{ex}= 370 nm, [DMPC] = 0.4 mM, [fisetin] = 4 μM.

membrane due to induced distortion in the packing of the head groups. However, at the more fluidic LC phase, the highly incorporated cholesterol molecules can reduce the internal volume of the bilayer, and consequently, the freedom of acyl chain movement tends to diminish, which results in a semifluidic nature of the membrane.[27,35,38,39] There is a progressive broadening of the phase transition behavior of the membrane with incorporation of cholesterol, and at 50 mol % cholesterol, almost elimination of the phase transition occurs.[21,35,38] These changes in the phase behavior of the membrane due to the presence of cholesterol have been studied at different temperatures using fisetin as the fluorescence molecular probe by carefully monitoring the fluorescence intensity of its phototautomer form (Figure 9).

Figure 9 shows the fluorescence intensity of the phototautomer form of fisetin with increasing cholesterol concentration and temperature. The plots clearly show a significant decrease in the fluorescence intensity with an increase in the cholesterol concentration at various temperatures. However, the decrease is comparatively large at lower temperature, which suggests significant phase modification of the membrane at its compact solid gel state. With the increase in cholesterol concentration, the membrane becomes increasingly fluidic at lower temperature, and this is in accordance with the known phase transition broadening (Figure 9). This results in an almost similar semifluidic nature of the membrane at higher cholesterol concentration over a wide range of temperatures.

Figure 10 shows that at a given temperature, with an increase in the mole % of cholesterol, there is a decrease in

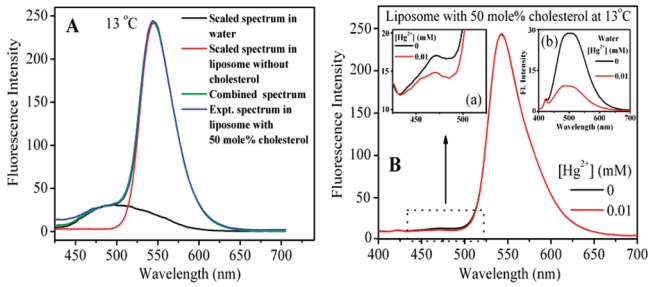


Figure 11. (A) Experimental and scaled emission spectra of fisetin in water and liposome. (B) Emission spectra of fisetin in the presence and absence of Hg^{2+} (0.01 mM) in DMPC vesicles with 50 mol % cholesterol at 13 °C. Inset (b) shows emission spectra of fisetin in the presence and absence of Hg^{2+} (0.01 mM) in water; λ_{ex}= 370 nm, [DMPC] = 0.4 mM, [fisetin] = 4 μM.

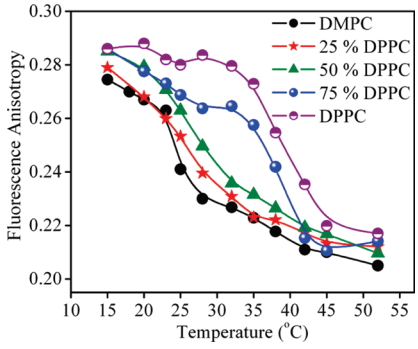


Figure 12. Plot of the variation of the phototautomer (λ_{ex} = 370 nm, λ_{em} = 545 nm) fluorescence anisotropy with temperature in liposomes prepared from DMPC and DPPC lipids; [fisetin] = 4 μM. The error in the data is within ±5%.

the phototautomer intensity accompanied by a small increase in the anion form intensity. The relative cholesterol-induced decrease in the phototautomer fluorescence intensity is found to be more at lower temperature as compared to that at higher temperature (Figure 9). There appears to be a cholesterol-induced expulsion of fisetin from the membrane to the aqueous solution, which is significant at lower temperature (Figure 10). Similar membrane-additive-induced expulsion of fluorescent membrane probes from the membrane bilayer has already been observed in the literature.[40,41] This expulsion, leading to a marginal decrease in the population of fisetin in the membrane with incorporation of cholesterol, can have implications in its antioxidant activity.

In order to ascertain whether the small shoulder appearing in the 450−500 nm range is due to the expelled fisetin anion emission, the measured fisetin anion emission spectrum in pure water with λ_{max} = 500 nm (Figure 2A) and pure fisetin tautomer emission from MLVs without cholesterol with λ_{max} = 545 nm (Figure 10) were added with suitable scaling factors, shown in Figure 11A. This combined spectrum closely matches with the experimental emission spectrum (Figure 10) of fisetin in MLVs with 50 mol % cholesterol, indicating that the small shoulder in Figure 10 indeed originates from fisetin anion fluorescence.

In order to confirm this assignment further, a fluorescence quenching study was performed with Hg^{2+} ion as the hydrophilic quencher (Figure 11B). In an aqueous medium, Hg^{2+} ion was

found to quench the fluorescence of the fisetin anion to an appreciable extent (Figure 11B, inset b). It is seen that with a 0.01 mM concentration of Hg^{2+} ion, the shoulder in the region of 450−500 nm is quenched appreciably, whereas the tautomer emission peak at 545 nm remains unquenched. Thus, it appears that with the incorporation of cholesterol, there is marginal expulsion of fisetin from the membrane to the aqueous phase.

Response of Fisetin Fluorescence to Mixed Lipid Systems. If a mixture of DMPC and DPPC lipids is used to constitute the liposome, then it is known that the phase transition temperature of the mixed lipid membrane changes with the composition of the lipid.[19,35,42] The phase transition temperature varies as 23 (pure DMPC), 28 (25% DPPC), 34 (50% DPPC), 38 (75% DPPC), and 42 °C (pure DPPC).[35,42] The sensitivity of the steady-state fluorescence anisotropy (r_{ss}) of the fisetin phototautomer form to the phase transition of mixed lipid systems was studied. Figure 12 shows the variation of the fluorescence anisotropy of the phototautomer with temperature and liposomes containing different compositions of DMPC and DPPC lipids. The spectral behavior of the mixed lipid system is found to be remarkably similar to that of pure DMPC and DPPC liposomes. However, the sigmoidal curve owing to fluorescence anisotropy of DMPC gradually shifts to higher temperature with an increase in the % of DPPC in the mixed lipid system, which represents the shift in the phase transition temperature to higher values with the increase in DPPC concentration, the trend being very similar to literature reports.[35,42]

All of these experimental results give clear substantiation that the fisetin molecule partitions into the DMPC vesicles and locates itself near the head group region of the lipid bilayer membrane. Being a fluorescent molecule, fisetin can also be competently used as an efficient membrane probe to study the membrane-related changes induced by temperature, external molecules, and additional phospholipids.

CONCLUSIONS

The intense green fluorescence of the phototautomer form of fisetin present in lipid bilayer membrane, the large Stoke's shift of ∼175 nm (λ_{ex} = 370 nm, λ_{em} = 545 nm) associated with this fluorescence, the absence of fisetin phototautomer fluorescence in water, the very weak photoanion fluorescence (λ_{ex} = 370 nm, λ_{em} = 500 nm) of fisetin in water, the high fluorescence anisotropy associated with the phototautomer fluorescence, and the reasonably large partition coefficient ($\sim(5.1 \pm 0.5) \times 10^5$) make fisetin an attractive candidate for a good fluorescent molecular probe, marker, and imaging dye. In the present study, it was seen that fisetin is a sensitive membrane probe for monitoring temperature-dependent phase change behavior of single as well as mixed lipid systems (DMPC and DPPC) and the effect of cholesterol on membrane properties. Further, with regards to the antioxidant activity of fisetin, the location in the head group region of the membrane away from the water-accessible region and the observation that it gets expelled from the membrane by cholesterol incorporation can have important implications.

ASSOCIATED CONTENT

Supporting Information. Fluorescence lifetime decay profiles of the phototautomer form of fisetin with an increase in concentration of CPC in THF and in DMPC vesicles at 15 and 35 °C. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Phone: (+91) 44-22574207. Fax: (+91) 44-22574202. E-mail: mishra@iitm.ac.in.

ACKNOWLEDGMENT

This research was supported by a research project funded by the Department of Science and Technology, Govt. of India. M.M. thanks IIT Madras for a research fellowship. The authors thank Dr. Amal Kanti Bera and Mr. Giriraj Sahu of the Biotechnology Department, IIT Madras, for the fluorescence microscopy image.

REFERENCES

(1) Middleton, M. D. E., Jr. *Pharm. Biol.* **1996**, *34*, 344–348.
(2) Cook, N. C.; Samman, S. *J. Nutr. Biochem.* **1996**, *7*, 66–76.
(3) Gordon, M. H.; Roedig-Penman, A. *Chem. Phys. Lipids* **1998**, *97*, 79–85.
(4) Benavente-García, O.; Castillo, J. *J. Agric. Food Chem.* **2008**, *56*, 6185–6205.
(5) Wang, L.; Tu, Y.-C.; Lian, T.-W.; Hung, J.-T.; Yen, J.-H.; Wu, M.-J. *J. Agric. Food Chem.* **2006**, *54*, 9798–9804.
(6) Chaudhuri, S.; Banerjee, A.; Basu, K.; Sengupta, B.; Sengupta, P. K. *Int. J. Biol. Macromol.* **2007**, *41*, 42–48.
(7) Sengupta, P. K.; Kasha, M. *Chem. Phys. Lett.* **1979**, *68*, 382–385.
(8) Kasha, M. *J. Chem. Soc., Faraday Trans.* **1986**, *82*, 2379–2392.
(9) Zhu, A.; Wang, B.; White, J. O.; Drickamer, H. G. *J. Phys. Chem. B* **2004**, *108*, 891–894.
(10) Demchenko, A. P.; Ercelen, S.; Roshal, A. D.; Klymchenko, A. S. *Pol. J. Chem.* **2002**, *76*, 1287–1299.
(11) Parthenopoulos, D. A.; McMorrow, D.; Kasha, M. *J. Phys. Chem.* **1991**, *95*, 2668–2674.
(12) Falkovskaia, E.; Sengupta, P. K.; Kasha, M. *Chem. Phys. Lett.* **1998**, *297*, 109–114.
(13) Guharay, J.; Dennison, S. M.; Sengupta, P. K. *Spectrochim. Acta, Part A* **1999**, *55*, 1091–1099.
(14) Funes, M.; Correa, N. M.; Silber, J. J.; Biasutti, M. A. *Photochem. Photobiol.* **2007**, *83*, 486–493.
(15) Sengupta, B.; Banerjee, A.; Sengupta, P. K. *FEBS Lett.* **2004**, *570*, 77–81.
(16) Selvam, S.; Mishra, A. K. *Photochem. Photobiol. Sci.* **2011**, *10*, 66–75.
(17) Guzzo, M. R.; Uemi, M.; Donate, P. M.; Nikolaou, S.; Machado, A. E. H.; Okano, L. T. *J. Phys. Chem. A* **2006**, *110*, 10545–10551.
(18) Banerjee, A.; Sengupta, P. K. *Chem. Phys. Lett.* **2006**, *424*, 379–386.
(19) Mohapatra, M.; Subuddhi, U.; Mishra, A. K. *Photochem. Photobiol. Sci.* **2009**, *8*, 373–378.
(20) Chaudhuri, S.; Pahari, B.; Sengupta, P. K. *Biophys. Chem.* **2009**, *139*, 29–36.
(21) New, R. R. C. *Liposomes, a practical approach*; Oxford University Press: New York, 1990.
(22) Lakowicz, J. R. *Principles of fluorescence spectroscopy*; Kluwer Academic, Plenum Publishers: New York, 1999.
(23) Huang, Z.; Haugland, R. P. *Biochem. Biophys. Res. Commun.* **1991**, *181*, 166–171.
(24) Gunsolley, J. C. *J. Dent.* **2010**, *38*, S6–S10.
(25) Arrigler, V.; Kogej, K.; Majhenc, J.; Svetina, S. *Langmuir* **2005**, *21*, 7653–7661.
(26) Varade, D.; Joshi, T.; Aswal, V. K.; Goyal, P. S.; Hassan, P. A.; Bahadur, P. *Colloids Surf., A* **2005**, *259*, 95–101.

(27) Subuddhi, U.; Haldar, S.; Sankararaman, S.; Mishra, A. K. *J. Photochem. Photobiol., A* **2008**, *200*, 381–387.
(28) Nakashima, K.; Yuda, K.; Ozaki, Y.; Noda, I. *Spectrochim. Acta, Part A* **2004**, *60*, 1783–1791.
(29) Gormin, D.; Sytnik, A.; Kasha, M. *J. Phys. Chem. A* **1997**, *101*, 672–677.
(30) Essmann, U.; Perera, L.; Berkowitz, M. L. *Langmuir* **1995**, *11*, 4519–4531.
(31) Petrache, H. I.; Tristram-Nagle, S.; Nagle, J. F. *Chem. Phys. Lipids* **1998**, *95*, 83–94.
(32) Terao, J.; Piskula, M.; Yao, Q. *Arch. Biochem. Biophys.* **1994**, *308*, 278–284.
(33) Arora, A.; Byrem, T. M.; Nair, M. G.; Strasburg, G. M. *Arch. Biochem. Biophys.* **2000**, *373*, 102–109.
(34) Ratty, A. K.; Sunamoto, J.; Das, N. P. *Biochem. Pharmacol.* **1988**, *37*, 989–995.
(35) Sujatha, J.; Mishra, A. K. *Langmuir* **1998**, *14*, 2256–2262.
(36) Pappayee, N.; Mishra, A. K. *Photochem. Photobiol.* **2001**, *73*, 573–578.
(37) Sujatha, J.; Mishra, A. K. *J. Photochem. Photobiol., A* **1997**, *104*, 173–178.
(38) Sankaram, M. B.; Thompson, T. E. *Biochemistry* **1990**, *29*, 10676–10684.
(39) Robinson, A.; Richards, W.; Thomas, P.; Hann, M. *Biophys. J.* **1995**, *68*, 164–170.
(40) Memoli, A.; Annesini, M. C.; Petralito, S. *Int. J. Pharm.* **1999**, *184*, 227–235.
(41) Benachir, T.; Lafleur, M. *Biochim. Biophys. Acta* **1995**, *1235*, 452–460.
(42) Shimshick, E. J.; McConnell, H. M. *Biochemistry* **1973**, *12*, 2351–2360.