Fluorescent molecular probes based on excited state prototropism in lipid bilayer membrane

Monalisa Mohapatra and Ashok K Mishra*

Department of Chemistry, Indian Institute of Technology Madras, Chennai 600036, India

mishra@iitm.ac.in

ABSTRACT

Excited state prototropism (ESPT) is observed in molecules having one or more ionizable protons, whose proton transfer efficiency is different in ground and excited states. The interaction of various ESPT molecules like naphthols and intramolecular ESPT (ESIPT) molecules like hydroxyflavones etc. with different microheterogeneous media have been studied in detail and excited state prototropism as a probe concept has been gaining ground.[1] The fluorescence of different prototropic forms of such molecules, on partitioning to an organized medium like lipid bilayer membrane, often show sensitive response to the local environment with respect to the local structure, physical properties and dynamics. Our recent work using 1-naphthol as an ESPT fluorescent molecular probe has shown that the incorporation of monomeric bile salt molecules into lipid bilayer membranes composed from dipalmitoylphosphatidylcholine (DPPC, a lung surfactant) and dimyristoylphosphatidylcholine (DMPC), in solid gel and liquid crystalline phases, induce appreciable wetting of the bilayer up to the hydrocarbon core region, even at very low (≤ 1 mM) concentrations of the bile salts. The incorporation and location of fisetin, an ESIPT molecule having antioxidant properties, in lipid bilayer membrane has been sensitively monitored from its intrinsic fluorescence behaviour.

Keywords: ESPT, Bile salt, Membrane hydration, Flavonoids

1. INTRODUCTION

The high sensitivity of fluorescent molecular probe-based techniques and multiple measurement parameters associated with fluorescence makes it a suitable method for studying biological systems and their interactions. Fluorescent molecular probes based on excited state prototropism (ESPT) are highly useful in obtaining structural and dynamical

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information on a variety of aggregate and organized systems such as polymeric gels, micelles, lipid bilayer membranes, cyclodextrin cavities, etc. [1, 2] 1-naphthol, hydroxyflavones and some related compounds have proven to be quite sensitive to the structure and organization of lipid bilayer membranes and various membrane dynamical properties. Membrane related changes often sensitively affect the excited state prototropic equilibrium of the probe molecule, which is reflected in its fluorescence parameters. Using 1-naphthol as a sensor, it has been possible to sense the membrane-related changes induced by additives like surfactants,[3] cholesterol,[4] bile salts[5, 6] etc.

Bile salts are steroids possessing hydrophilic and hydrophobic domains in opposite faces which provide facial polarity to the molecules. To understand many important biological processes like solubilization of cholesterol, lipids and fat-soluble vitamins in living organisms, the surfactant behaviors of bile salts have been extensively studied.[7, 8] In a number of physiological processes, different types of interactions take place between bile salts and phospholipid bilayers, e.g. during liposomal drug delivery in hepatobiliary systems, liposomes remain exposed to bile salts.[9, 10] This explains the importance of a study of bile salt - liposome interactions. Bile salts form different states of aggregation at different concentrations and temperatures. The micellar forms of bile salts at higher concentrations are known to significantly alter the lipid bilayer organization.[9–11] This work focusses on the bile salt – liposome interaction at sub-micellar concentration of bile salts. Given the amphiphilic nature of bile salt molecules, membrane perturbation effects at low concentrations at these concentrations, bile salts (expected to be in monomeric form) binds efficiently to the membranes enhancing the spontaneous rate of intervesicular phospholipid transfer process.[12, 13, 14]

Phosphatidylcholines are the most important among the vesicle forming phospholipids in biological systems. The present work is focused on the interaction of vesicles composed of phosphatidylcholines and submicellar concentrations of naturally occurring bio-surfactants (bile salts) using fluorescence parameters of 1-naphthol, an ESPT fluorescent molecular probe and 1, 8-anilino naphthalene sulphonate (ANS), a polarity sensitive probe. This follows our recent study on certain aspects of the problem, which indicated appreciable hydration of the lipid bilayer up to the hydrocarbon core region[5, 6]. Fisetin is among the most important naturally occurring plant flavonoids which shows anti-inflammatory, anti-cancer, anti-allergic, anti-AIDS and anti-oxidant properties[15] and is an active excited state intramolecular proton transfer (ESIPT) molecule. Depending on the surrounding microenvironment, fisetin exists in different prototropic forms. Since the antioxidant properties of flavonoids in lipid bilayer membrane depend on their location in bilayer, the incorporation and possible location of fisetin in lipid bilayer membranes have been discussed using its intrinsic fluorescence behaviour.

2. METHODOLOGY

2.1. MATERIALS

1-Naphthol purchased from SRL, India was sublimed and used after checking its purity. ANS, Fisetin, DMPC and DPPC were purchased from Sigma Chemical Co. (Bangalore, India) and used as received. NaDC, NaC and cetylpyridinium chloride (CPC) were purchased from SRL, India and used as received. All the solvents used were of spectral grade. Triple-distilled water, prepared using alkaline permanganate solution, was used for the experiments.

2.2. LIPOSOME PREPARATION

For this work, small unilamellar vesicles were prepared by the ethanol injection method.[16] 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 probe and equilibrated for 30 min at 50 ^{o}C (above phase transition temperature of DMPC and DPPC). The percentage of ethanol in the solution was < 1% (v/v). Throughout the experiments, the probe-to-lipid molar ratio was kept constant at 1:100 (4 μM probe, 0.4 mM lipid). All experiments were performed with freshly prepared solutions of both probe and liposome.

2.3. INCORPORATION OF BILE SALTS

Stock solutions of NaDC and NaC were prepared in triple-distilled water at neutral pH. The solutions were prepared by adding the desired volume of bile salt stock of appropriate concentration to the liposome solutions at 50 ^{o}C (LC phase). The solutions were equilibrated for 2 h before analysis. All the experiments were performed with freshly prepared solutions of bile salts.

2.4. FLUORESCENCE MEASUREMENTS

Fluorescence measurements (steady state intensity and anisotropy) were carried out with a Hitachi F-4500 and Fluoromax-4 spectrofluorometer. Emission spectra were recorded by fixing excitation wavelength at 290 nm (1-naphthol) and 370 nm (fisetin, ANS). Temperature was controlled by circulating water through a jacketed cuvette holder from a refrigerated bath (Julabo, Germany).

2.5. FLUORESCENCE LIFETIME MEASUREMENTS

Fluorescence lifetime measurements were carried out using Horiba Jobin Yvon TCSPC lifetime instrument in a time-correlated single-photon counting arrangement. Nano-LEDs of 295 and 370 nm were used as the light source. The pulse repetition rate was set to 1 MHz and the instrumental full width half maximum of the 370 nm LED, including the detector response was ca. 1.1 ns and the instrumental full width half-maximum of the 295 nm LED, including the detector response was ca. 800 ps.. The instrument response function was collected using a scatterer (Ludox AS40

colloidal silica). The decay data were analyzed using IBH software. A value of $\chi 2$, $0.99 \leq \chi 2 \leq 1.2$ was considered as a good fit, which was further judged by the symmetrical distribution of the residuals.

3. RESULTS AND DISCUSSION

The emission spectra of 1-naphthol (λ_{ex} = 290 nm, λ_{em} = 370 and 465 nm) is shown in figure 1A and B, with varying concentrations of NaDC in DMPC vesicles at 15 °C (SG phase) and 35 °C (LC phase), respectively. Figure 1C and D represents the same with varying concentrations of NaC. The concentration of bile salts was varied between 0.05 and 1 mM (well below their CMC), keeping the DMPC concentration constant at 0.4 mM.

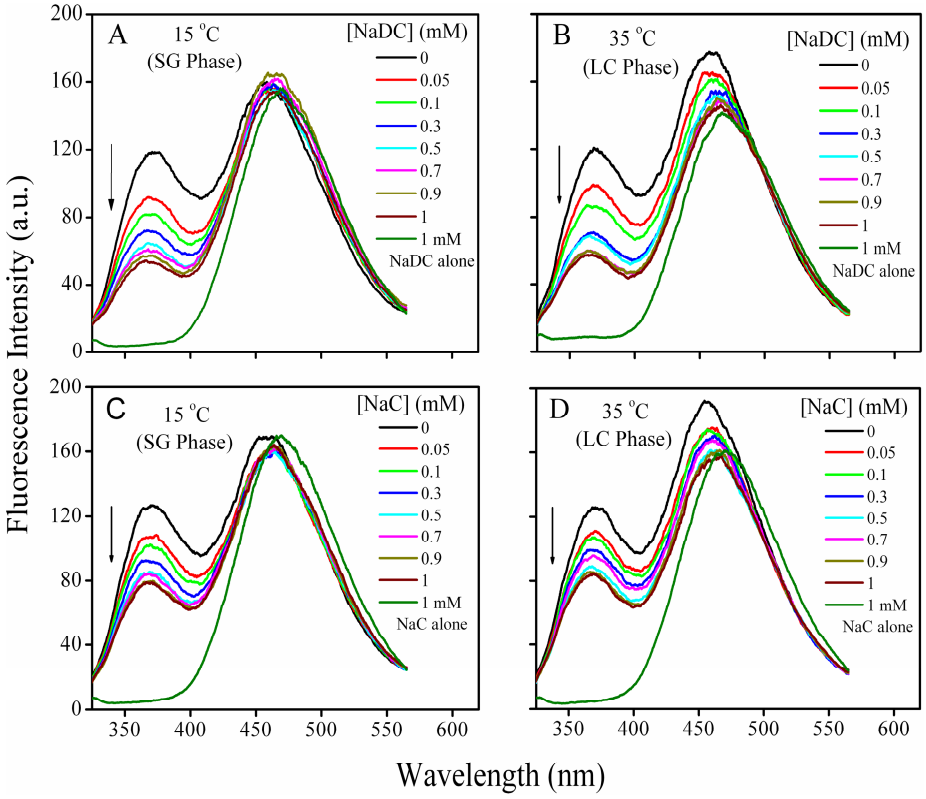

Figure 1. Response of the fluorescence intensity of 1-naphthol to the changes induced by (A and B) NaDC and (C and D) NaC in DMPC vesicles. (λ_{ex} = 290 nm, λ_{em} = 370 and 465 nm). [DMPC]= 0.4 mM, [1-naphthol] = 4 μM.

Depending on the medium of study, 1-naphthol remains in neutral (ROH*) and anionic (RO^-*) emitting forms which are in equilibrium with one another. In aqueous medium, light-emitting form is the RO^-* (λ_{ex} = 290 nm, λ_{em} = 470 nm), whereas, in liposome medium, emissions occur from both ROH* (λ_{ex} = 290 nm, λ_{em} = 370 nm) and RO^-* (λ_{ex} = 290 nm,

λ_{em} 465 nm) forms. Literatures suggests that, in liposome medium the ROH* and RO^-* form emissions arise from the 1-naphthol molecules present in the water-inaccessible hydrophobic core and at the water-accessible hydrophilic surface of the lipid bilayer, respectively.[4, 17] In bile salt (1 mM) solution (without liposome), there is absence of ROH* emission (Figure 1). However, in the liposome medium there is a decrease in intensity of ROH* with increase in bile salt concentration, both in the SG and LC phase. The RO^-* emission arises from the unpartitioned 1-naphthol from bulk water as well as from the RO^-* originating from 1-naphthol present in the membrane interface, hence, the fluorescence of RO^-* does not purely reflect the prototropic changes of membrane bound 1-naphthol. The RO^-* emission from the aqueous phase and the membrane bound 1-naphthol can be distinguished from their emission wavelengths and lifetime values. Since ROH* emission originates only from the membrane bound 1-naphthol, the changes in fluorescence of ROH* can be directly ascribed to the changes in membrane due to its interaction with bile salt.

For an ESPT to occur, a cluster of four water molecules acts as a base which is responsible for accepting the dissociated proton.[18] Since the water cluster is the proton accepter, any change in the hydration of membrane is reflected in the changes in neutral form fluorescence. As 1-naphthol is known to be distributed among the core as well as interface regions, it can sense the extent of hydration across the membrane. This has been found in submicellar concentration of bile salt-liposome interaction study, where neutral form fluorescence and lifetime of 1-naphthol are very sensitive to the bile salt induced hydration of the bilayer membrane.[5, 6] It has been observed that with increase in bile salt concentration a decrease in neutral form fluorescence intensity as well as life time occurs. Initially, it was thought that these observed neutral form parameter changes may be due to either of three possible reasons from the bile salt – membrane interaction; (i) expulsion of 1-naphthol from membrane to the solution; (ii) population redistribution of 1-naphthol within the membrane and (iii) permeation of water into the bilayer membrane. Later, from the detail lifetime studies and proton transfer rate constant data, it was concluded that the first two possible reasons were not likely the reasons for the observed spectral changes. Rather, the observed spectral changes were because of the water of hydration up to the core of the bilayer membrane. This is found to be due to the carriage of water of hydration in to the bilayer by the bile salts.

For ROH* (λ_{ex} = 295 nm, λ_{em} = 370 nm) in liposome medium, decay dynamic studies were carried out with varying bile salt concentrations at both the phase states (LC and SG). The decay of ROH* fluorescence in DMPC liposome medium was found to be biexponential with a short lifetime component (τ_s) of 3.0_9 ns and a long lifetime component (τ_l) of 7.2_9 ns in SG phase.[6] The corresponding values in the LC phase were 2.8_4 ns (τ_s) and 6.1_1 ns (τ_l). As per the two-state distribution model involving the membrane interface and hydrocarbon core, the observed biexponential fluorescence decay of ROH* was expected.[4] It was observed that with the addition of bile salts lifetime values of τ_s and τ_l of ROH* decreases with almost no change in the amplitudes of the corresponding lifetime components.

The concept of bile salt induced hydration of lipid bilayer membrane in presence of submicellar concentrations of bile salt is further supported by the fluorescence parameters of a polarity sensitive probe, ANS. It is very less fluorescent in

water, but becomes highly fluorescent with a blue shift in emission maximum in lesser polar solvents or on binding to hydrophobic systems.[19] The fluorescence properties of ANS are sensitive to the polarity of the microenvironment. Thus it has been widely used as polarity indicator in many biological systems. Figure 2A and B shows the response of ANS (λ_{ex} = 370 nm, λ_{em} = 470) fluorescence intensity with varying concentrations of NaDC and NaC, respectively, in DPPC vesicles at 30 °C (SG phase).

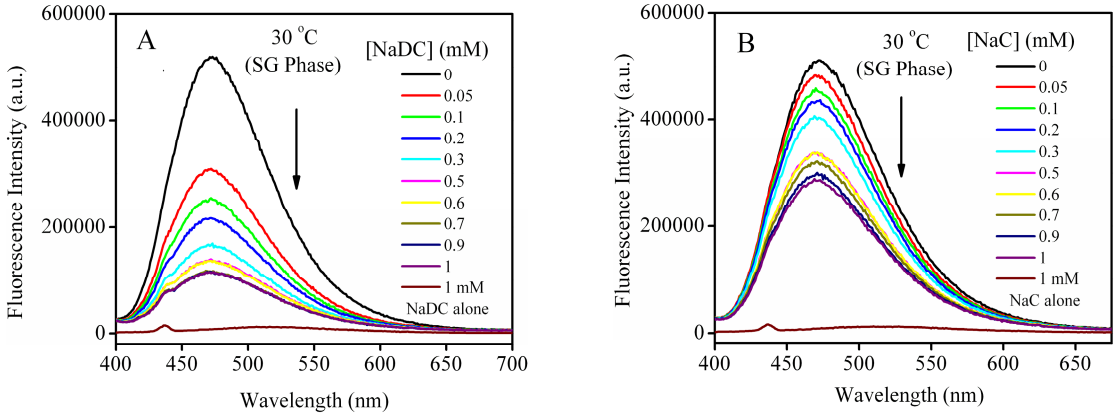

Figure 2. Response of ANS fluorescence intensity with increase in (A) NaDC and (B) NaC concentrations from 0.05 to 1 mM at 30 °C in DPPC vesicles. (λ_{ex} = 370 nm, λ_{em} = 470 nm). [DPPC] = 0.4 mM, [ANS] = 4 μM.

It has been observed that, with increase in bile salt concentrations there is a decrease in ANS fluorescence intensity. It is known that, with increase in water content fluorescence intensity of ANS decreases.[20] Thus, the drop in intensity may be attributed to the bile salt induced hydration of the lipid bilayer membrane at submicellar concentration of the bile salts. The drop in ANS fluorescence intensity is found to be more in presence of NaDC as compared to NaC. This further agrees with the fact that the wetting of lipid bilayer in presence of NaDC is more as compared to NaC.[5] But the decrease in ANS fluorescence intensity also could be due to expulsion of ANS (almost non-fluorescent in water) from the bilayer to the aqueous phase. Since steady state fluorescence study of ANS is inadequate to find out the possible reason for the drop in fluorescence intensity, fluorescence lifetime study has been carried out. Figure 3 shows the fluorescence lifetime decay of ANS (λ_{ex} = 370 nm, λ_{em} = 470) with varying bile salt concentrations in DPPC vesicles.

The bi-exponential decay profile of ANS in liposome suggests local heterogeneity in its distribution. From the decay profile it has been observed that with increase in bile salt concentrations there is a drop in fluorescence lifetime. If there would have been expulsion of ANS from the membrane, fluorescence lifetime of ANS would not have changed in presence of bile salt. Since there is a drop in fluorescence lifetime with increasing bile salt concentrations, the possible reason of expulsion of ANS might not be the reason for the drop in fluorescence intensity. This further supports the concept of bile salt induced lipid bilayer hydration in presence of submicellar concentrations of bile salt. The drop in fluorescence lifetime is more in presence of NaDC as compared to NaC. Being more hydrophobic NaDC interacts more efficiently with the lipid bilayer membrane resulting in a better bilayer hydrating ability as compared to NaC.

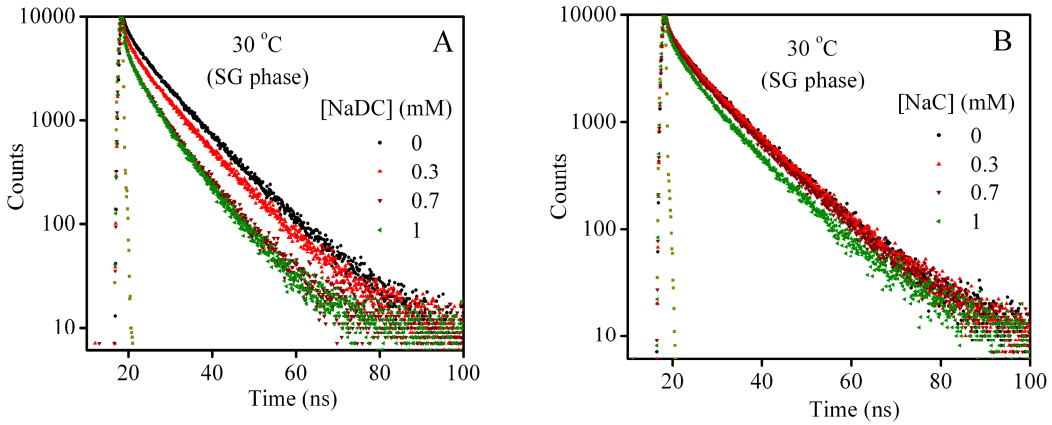

Figure 3. Fluorescence lifetime decay of ANS with varying (A) NaDC and (B) NaC concentrations at 30 °C in DPPC vesicles. (λ_{ex} = 370 nm, λ_{em} = 470 nm). [DPPC] = 0.4 mM, [ANS] = 4 μM.

Unlike ESPT molecules like 1-naphthol, for which the predominant light emitting form is the neutral form, in case of ESIPT molecules like 3-hydroxyflavone and fisetin, the predominant light emitting form is the phototautomer form in liposome medium. Previous studies on photophysical behaviour of 3-hydroxyflavone in DMPC liposome membrane suggests that the fluorescence of phototautomer form originates from the 3-hydroxyflavone molecules present at the hydrocarbon core of the bilayer membrane.[21] However, the fluorescence of fisetin phototautomer form comes from the membrane bound fisetin molecule, which is suggested to be near the bilayer interface.[22, 23] Here the incorporation and possible location of fisetin in lipid bilayer membranes have been discussed using fluorescence behavior of its phototautomer form.

Figure 4 represents the excitation and emission spectra of fisetin in DMPC liposome membrane, whereas the inset shows the excitation and emission spectra of fisetin in water. From the experimental results the following points has been observed; (i) almost 50-fold enhancement in fluorescence intensity of phototautomer (λ_{ex} = 370 nm and λ_{em} = 545 nm) in liposome as compared to the photoanion (λ_{ex} = 370 nm and λ_{em} = 500 nm) in water, (ii) a remarkable shift in the fluorescence maxima ca. 45 nm on going from water (500 nm) to liposome medium (545 nm) and (iii) a high fluorescence anisotropy value (0.27) of phototautomer in liposome than photoanion in water (0.06), which confirms the possible incorporation of fisetin in to the liposome membrane.

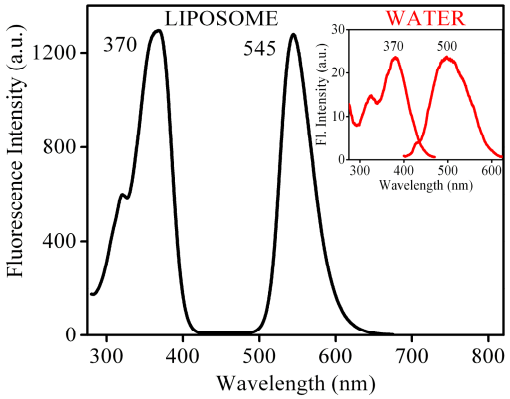

Figure 4. Excitation (λ_{em}= 545 nm), emission (λ_{ex}= 370 nm) spectra of fisetin in DMPC liposome at 35 ^{o}C and (inset) excitation (λ_{em}= 500 nm) emission (λ_{ex}= 370 nm) spectra of fisetin in water at neutral pH. [Fisetin] = 5 µM, [DMPC] = 0.4 mM

In order to understand the microenvironment around fisetin, it is important to know about its location in the bilayer membrane. The probable location of fisetin in bilayer has been confirmed from quenching experiment using cetylpyridinium chloride (CPC) as a quencher.[23] The positively charged pyridinium group of CPC is situated near the negatively charged phosphate group of the phospholipid molecule. Hence, CPC is supposed to quench selectively the fraction of fisetin molecules located near the interfacial site of the bilayer.[24] Figure 5 shows the plot of variation of fisetin fluorescence intensity in DMPC vesicles with varying the amount of CPC, at 15 ^{o}C and 35 ^{o}C. The phototautomer fluorescence intensity decreases gradually with the addition of CPC at both the phase states of the bilayer. From the selective quenching of phototautomer fluorescence intensity, the exact location of fisetin molecule in lipid bilayer membrane was confirmed to be near the water-accessible interfacial region.[23]

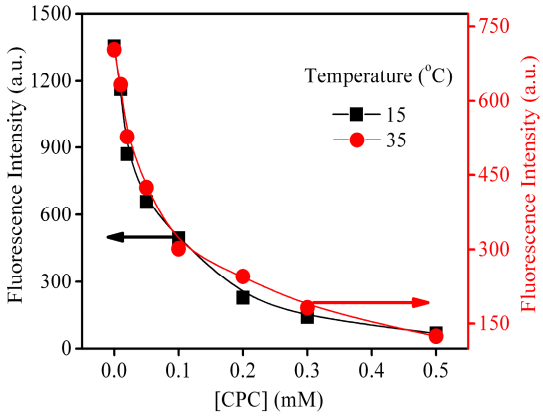

Figure 5. Plot of variation of fisetin fluorescence (λ_{max}= 545 nm) intensity with varying amount of CPC, at 15 ^{o}C (SG phase) and 35 ^{o}C (LC phase) in DMPC vesicles. [CPC] = 0 to 0.5 mM, [Fisetin] = 4 µM, [DMPC] = 0.4 mM

The photophysical properties of fisetin in lipid bilayer membrane like large Stokes' shift, gaussian spectral profile, emission in green region and ca. 50- fold enhancement in phototautomer intensity with respect to photoanion in water makes it a suitable molecule to be used as a marker in biological systems and in fluorescence imaging studies.[23]

4. CONCLUSION

In the interaction study of submicellar concentrations of bile salt and lipid bilayer membrane, fluorescence parameters such as intensity, transition maximum, and decay parameters of ESPT probe 1-naphthol and polarity probe ANS are found to be sensitive to the bile salt induced hydration of the membrane, which might have further implications in many biological studies. ESIPT molecule fisetin do partition into the DMPC vesicle, locates itself near the water-accessible interfacial region of the lipid bilayer membrane and found to be a suitable molecule for fluorescence imaging studies.

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