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DOI: 10.1039/C8CP00492G

Probing the Temperature Dependent Changes of the Interfacial Hydration and Viscosity of Tween20:Cholesterol(1:1) Niosome Membrane using Fisetin as a Fluorescent Molecular Probe

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Abstract

A detailed photophysical study of fisetin in Tween20:cholesterol(1:1) niosome membrane has been carried out. Fisetin is found to partition well into the Tween20:cholesterol(1:1) niosome membrane at low temperature ($K_p = 2.7 \times 10^4$ M^{-1} at 10 ^oC). Cetylpyridinium chloride (CPC) quenching study confirms the location of fisetin molecules in the interfacial domain of Tween20:cholesterol(1:1) niosome membrane. The emission from the prototropic forms of fisetin (neutral form, excited state anion, ground state anion and phototautomer form) is found to sensitively reflect the local heterogeneities in Tween20:cholesterol(1:1) niosome membrane. The shifting in anionic emission maximum with variation in temperature shows the sensitivity of fisetin towards the water accessibility at the interfacial domain of Tween20:cholesterol(1:1) niosome membrane. Zeta potential value confirms that there is no role of surface charge on the multiple prototropism of fisetin in Tween20:cholesterol(1:1) niosome membrane. The microviscosity changes with temperature, as reflected in fluorescence anisotropy values of FT*, give information about the temperature induced changes in the motional resistance offered by the interfacial domain of the niosomal membrane to small molecules. The temperature dependent fluorescence lifetime study confirms the distribution of FT* in the two different sites of niosomal interfacial domain i.e water deficient inner site and water

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accessible outer site. This heterogeneity in distribution of FT* is further confirmed through time resolved fluorescence anisotropy decay resulting in two different rotational time constants (faster component of ~ 1.04 ns originates from water accessible outer site and slower component of ~16.50 ns originates from water deficient inner site).The interfacial location of fisetin in Tween20:cholesterol(1:1) niosome membrane has an important implication with regards to antioxidant activity as confirmed from DPPH radical scavenging study.

Introduction

Fluorescent molecular probes based on excited state protoptropism are known to sensitively respond to the local environments of a microheterogeneous medium through various fluorescence spectral parameters of different excited state prototropic forms [1]. The excited state prototropism is known to be affected by temperature, in presence of additives, pH of the medium etc in an organized medium. For the biological application point of view it is worthy to study the effect of all parameters (temperature, presence of additives, and variation of medium pH) on lipid bilayer membrane, niosome membrane, cyclodextrin, micelle, reverse micelle etc. So it is more helpful to use excited state proton transfer (ESPT) and excited state intramolecular proton transfer (ESIPT) molecules to understand the microheterogeneity of different organized media. Fluorophores like 1-naphthol, 2-naphthol, 4-chloro-1-naphthol etc come under ESPT category whereas fisetin, quercetin, hydroxyflavone etc come under ESIPT category [1]. Like other ESIPT molecules, fisetin (scheme 1C) [2] also gives multiple emission bands in different organized media depending upon its surrounding environment [3]. Due to the presence of multiple equilibria between different emitting species, fisetin possesses four different types of emission

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namely ground state anionic emission (λ_{ex} = 418 nm, ~ 490 nm), excited state anionic emission (λ_{ex} = 370 nm, λ_{em} = ~ 490 nm), neutral form emission (λ_{ex} = 370 nm, λ_{em} = 410 - 430 nm) and red shifted phototautomer emission (λ_{ex} = 370 nm, λ_{em} = ~ 535 nm) [4,5]. The ESIPT process of fisetin is more prominent in organized media with a hydrophobic domain whereas ground state anion emission formation is possible only when there is a proton acceptor in ground state or if the medium is alkaline [4]. Fisetin is a polyphenolic compound present in onions, apples and tea [4] and is known to have anti-cancer, anti-inflammatory and anti-microbial activities [6-9]. The ESIPT process of fisetin has been studied in various microheterogeneous media like cyclodextrin [10], lipid bilayer membrane [11], polymer [12], micelle [4] and reverse micelle [13]. Thus the fluorescence of fisetin offers interesting possibilities of looking at the niosomal membrane domain.

Selvam et al. have studied the multiple prototropism of fisetin in sodium cholate and related bile salts media to establish the drug carrier activity of micelle for fisetin and other pharmaceutically important flavonoids [4]. Mohapatra et al. have studied the photophysical behavior of fisetin in DMPC multilamellar vesicle in a greater detail [11]. They have explained the structural changes in lipid bilayer membrane as a function of temperature and cholesterol concentration by using the sensitivity of fisetin. Guzzo et al. have studied the complaxation of fisetin with cyclodextrin (CD) as fisetin and CD are known to be a potential therapeutic drug and a good drug delivery system [10]. The biological importance of fisetin prompted us to study its photophysics in niosome membrane in greater details.

Organized media have a major role in modulating the photophysics of ESPT and ESIPT molecules [1]. The ESPT and ESIPT processes are known to be affected by the confined microenvironments of cyclodextrins, lipid bilayer membrane, proteins, polymers, micelles,

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microemulsions etc. Besides all these organized media, the non ionic surfactant formed vesicles niosomes are also much effective for the modulation of photophysics of ESPT and ESIPT molecules. These vesicles were first reported by Handjani-Vila et al. in the 70s [14]. It has both biological and industrial application because of its amphiphilic nature. It can solubilize both hydrophilic and lipophilic drugs through encapsulation either in aqueous layer or in vesicular membrane [15]. Like liposomes, niosomes are also efficient drug carrier because of its low toxicity, high stability and biocompatibility nature [16]. Basically the constituents of niosomes are nonionic surfactants (Tween, brij, Triton-X), cholesterol and some stabilizing additives. Among tween surfactants tween20 (scheme 1B) is used in the present study because of its low cost, biocompatibility and low toxicity along with cholesterol (scheme 1C) [17].

In our previous study we have reported that Tween20:cholesterol(1:1) niosomal interfacial domain gets hydrated with increase in temperature which was understood through different fluorescence parameters of 1-naphthol [17]. So far there is no report on the use of fluorescence anisotropy changes of 1-naphthol in probing microheterogeneous media. In contrast, fisetin fluorescence anisotropy is known to sense organizational changes in bilayer membranes [11], bile salt micelle [4], PF127 hydrogel [18], β-cyclodextrins [3] etc. Thus, fisetin as a fluorescent molecular probe appears particularly suitable for this purpose. The other attractive feature of fisetin as a molecular probe is that its intense phototautomeric form fluorescence observes mainly in polar but water deficient environment [4,5,11] such an environment is a defining feature of niosomal membrane.

Mohapatra et.al have reported that fisetin molecules reside in the interfacial domain of lipid bilayer membrane of liposomes [11]. They have measured the fluorescence anisotropy

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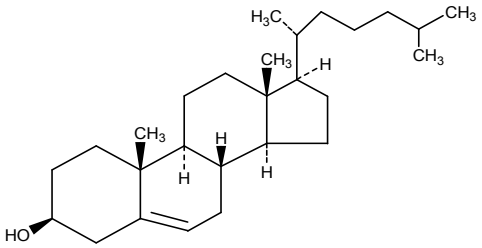
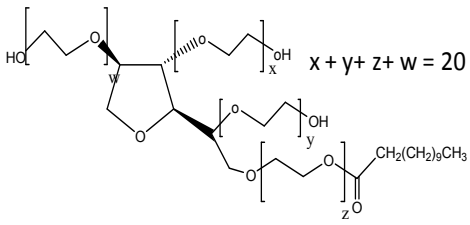
of fisetin tautomeric species to understand the membrane permeability with variation in temperature. Niosomes are similar to liposomes having a lumen and distinct bilayer morphology of the membrane. Thus fisetin could be an appropriate molecular probe to understand the membrane properties like the microviscosity, membrane hydration etc and their temperature dependence. This is the main objective of this study. Another interesting characterstics of fisetin is that it has antioxidant activity. In this study we have reported the efficiency of scavenging activity of fisetin in Tween20:cholesterol(1:1) niosome membrane using DPPH radical as a scavenger.

The main objectives of this study include: (i) to address fisetin as a good, sensitive fluorescent molecular probe for Tween20:cholesterol(1:1) niosome membrane, (ii) to understand the Tween20:cholesterol(1:1) niosomal membrane properties (microviscosity, membrane hydration) and their temperature dependence as it has pharmaceutical importance due to its low cost, higher chemical stability and easy to handle, (iii) to determine the efficiency of radical scavenging activity of fisetin in Tween20:cholesterol(1:1) niosome membrane.



(A) Cholesterol



(B) Tween20

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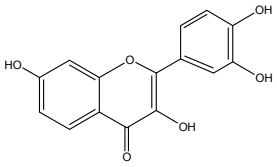
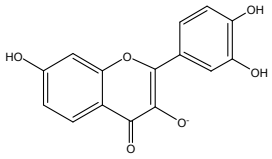
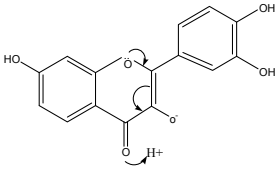


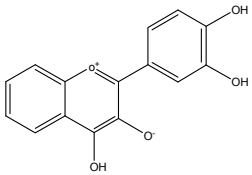
Neutral Form
λ_{ex} = 370 nm, λ_{em} = 400-430 nm



Ground State anion Form
λ_{ex} = 418 nm, λ_{em} = ~ 490 nm



Excited State anion
λ_{ex} = 370 nm, λ_{em} = ~ 470 nm



Phototautomer Form
λ_{ex} = 370 nm, λ_{em} = ~ 535 nm

(C) Different emissions of fisetin

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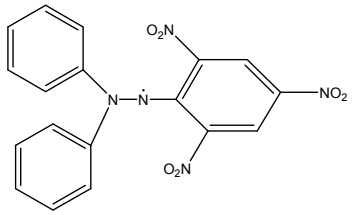

(D) 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH)

Scheme 1: Chemical structure of (A) cholesterol, (B) tween20 (C) fisetin and (D) DPPH

Materials and Methods

Materials **-** Fisetin was purchased from sigma-aldrich, Bangalor India.tween20 (TW20) and cholesterol were purchased from Merck chemicals. 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH) was purchased from TCI chemicals. Spectroscopic grade solvents (Chloroform, Methanol) and phosphate buffer (pH 6.8) were used for the

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preparation of both stock and experimental solution. Triple-distilled water, prepared by using potassium permanganate and sodium hydroxide was used throughout the experiments.

Methods –The size of the TW20:cholesterol(1:1) niosomes were measured by using dynamic light scattering (DLS) instrument (Malver zetasizer nano series) having 632.8 nm laser source (as excitation light) and 1 cm path length. The scattering angle was kept as 90^{o} for this measurement. Fluoromax-4 fluorescence spectrophotometer was used for the measurement of both fluorescence intensity and fluorescence anisotropy. Fluorescence lifetime and fluorescence anisotropy decay measurements were performed by using Horiba Jobin Yvon TCSPC lifetime instrument having a time correlated single photon counting arrangement. 370 nm LED was used as the excitation source for both the measurements. The pulse repetition rate was set to 1 MHz and the detector response time is less than 1 ns. The instrument response function was collected by using LUDOX AS40 colloidal silica solution (a scatterer). Lifetime data were analyzed by using IBH software. Decays were fitted by keeping the value of $\chi 2$ in between 1.04-1.18 having symmetrical distribution of residuals. The average fluorescence lifetime (τ_{avg}) values were calculated by the following equation (1) [11]

$$\tau_{avg} = \left(\sum_{i=1}^{n} \alpha_i \tau_i\right) \Big/ \left(\sum_{i=1}^{n} \alpha_i\right) \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Where τ_i is the individual lifetime with corresponding amplitude α_i.

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Lifetime distribution fitting was done by using same IBH software, following the previous report.[17]

For the analysis of the time-resolved anisotropy decay IBH software was used which is based on the reconvulation technique using a nonlinear least-squares method. The study was carried out by measuring the polarized fluorescence decays $I_{||}$ (t) and $I_{\perp}$(t). The anisotropy decay function r(t) was obtained from this two polarization values by using equation (2) [19, 20].

$r(t) = \frac{I_{\parallel}(t) - GI_{\perp}(t)}{I_{\parallel}(t) + 2GI_{\perp}(t)}$ ..... (2) Here G is the correction factor, for this study value of G is 0.6.

TW20 Micellar Solution Preparation - The TW20 micellar solutions have prepared by varying the concentration of TW20 (0-2 mM) in triple distilled water having fisetin concentration (5 μM) constant. All the experimental solutions have kept for 2 hr equilibration before carried out all the experiments.

Niosomes Preparation - In this present work we have prepared TW20:cholesterol(1:1) niosome using thin film hydration method as available in literatures [16,17,21,22]. TW20 and cholesterol (1.25 mM) were dissolved in chloroform:methanol (2:1),(v/v) mixture. The solvent (mixture of TW20:cholesterol (2mL:2mL)) was evaporated by using a rotary evaporator, and then the round bottom flask was allowed to keep in vacuum for one hour to remove the residual solvent if any left. Required volume of methanolic solution of fisetin (5 μM) was added to the solvent mixture of TW20:cholesterol (2mL:2mL) before evaporation. After complete dryness, required volume of phosphate buffer (5 mL) having pH 6.8 was added to hydrate the lipid film with vigorous shaking at 60 ^{o}C. Then the

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dispersion was sonicated for 10 minute by using a probe sonicator. Sonicated lipid dispersion was centrifuged to remove unwanted large particles and free surfactants.1.25 mM of both TW20 and cholesterol was used in this experiment which is above the critical micellar concentration (CMC) of TW20 surfactant. To get a better spherical association same amount (1:1) of cholesterol and TW20 was used for the preparation of niosome use in this experiment.

Microviscosity Calculation - The microviscosity in an organized medium can be calculated by using Perrine's equation (3), which involves fluorescence anisotropy and fluorescence lifetime value [23, 24]

$$\frac{r_0}{r_{ss}} = 1 + C(r)\frac{T\tau}{\eta} \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Here r_o represents the limiting anisotropy value of probe in the absence of any depolarizing process like rotational diffusion and energy transfer whereas r_{ss} is the measured steady state fluorescence anisotropy. T is the temperature in Kelvin, τ is the fluorescence lifetime of the probe in second and C(r) represents the molecular shape and location of the transition dipole of the rotating fluorophore. The simplified form of equation (3) for a heterogeneous microenvironment can be written as [25]

$$\eta = \frac{C(r)T\tau r_{ss}}{\delta_r} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)(here\ \delta_r = r_o - r_{ss})$$

By substituting all the parameters the microviscosity of the niosome membrane was calculated with variation in temperature.

DPPH Scavenging Activity - The radical scavenging activity of fisetin in TW20:cholesterol(1:1) niosome membrane has studied by using DPPH as a free radical. It

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is well known that DPPH is a good radical scavenger [26, 27, 28]. An ethanolic solution of 50 μM DPPH was added to fisetin (5 μM) loaded niosome formulation. The absorption spectrum of the above formulation (DPPH-fisetin loaded niosome) was recorded by using Shimadzu-UV-spectrophotometer at room temperature as a function of time. The scavenging activity of DPPH was calculated by using equation (5) [28]

$$\text{DPPH scavenging activity (\%)} = \left(1 - \frac{A_{sample} - A_{blank}}{A_{control}}\right) \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots (5)$$

Here A_{sample} is the absorbance value of DPPH in fisetin loaded niosome as a function of time, $A_{control}$ is the absorbance value of DPPH in distilled water and A_{blank} is the absorbance value of fisetin in ethanol.

Results and discussions

Characterization of Niosomes

DLS study

The characterization technique used for this studied to characterize TW20:cholesterol(1:1) niosomes is dynamic light scattering (DLS) study. Figure 1 shows the size distribution histogram of TW20:cholesterol(1:1) niosomes as obtained from DLS measurement at 25 ^{o}C. The average size of the niosome is found to be ~ 124 nm which matches with the size of the literature reported value (100 – 200 nm) [16,17,21,22].

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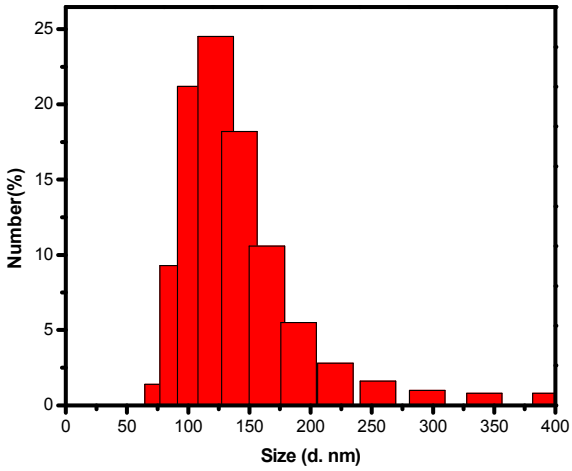

Figure 1: DLS histogram plot for TW20:cholesterol(1:1) niosome at room temperature. The narrower distribution and the number percentage maximum at 124 nm indicate the formation of uniform nano vesicles.

Photophysics of Fisetin in TW20 Micelle

A preliminary study of fisetin in TW20 micelle has carried out to understand the photophysics of fisetin in TW20:cholesterol (1:1) niosome membrane. An intense peak observes at ~ 370 nm in uv-visible absorption spectra of fisetin in TW20 micellar medium (figure S1). This peak at ~370 nm corresponds to the fisetin neutral species similar to those observed in non-polar solvent and also in NaC bile salt medium [4]. As the concentration of TW20 increases the peak intensity also increases, this is because of the solubalization of fisetin in TW20 micellar medium.

In emission spectra, at lower concentration of TW20, fisetin possesses a broad structureless band at λ_{em} ~ 498 nm, which is attributed to the overlapping of both anionic and tautomeric emission as reported in literature [5]. A remarkable change in the emission behaviour of fisetin has observed from 0.02 mM of TW20. Presences of two iso-emissive

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points indicate that three prototropic emissions of fisetin are in equilibrium with each other (neutral emission, excited state anionic emission, tautomeric emission). With increase in concentration of TW20 the tautomeric and neutral intensity increase with a concomitant decrease in anionic intensity,(figure S2 A) this indicates that there is an increase in hydrophobocity of the medium with increase in TW20 concentration, as tautomeric and neutral emission of fisetin originate from polar but water deficient environment [5]. This is also observed from the intensity ratio plot (figure S2 C). Emission maximum of phototautomer emission shifted from (525 - 535 nm) (figure S2 A).The wave number plot (cm^{-1}) indicates the CMC of TW20 (figure S2 B). It indicates that upto CMC there is a shifting in tautomeric emission maximum after that it remains constant; this point of inclination gives the value of CMC which is closely similar to the reported value [29]. The decrease in anionic intensity indicates that there is a decrease in polarity of the fisetin environment as process of micellization start (figure S2 A). The anisotropy value of fisetin (λ_{ex} = 370 nm, λ_{em} = 535 nm) at lower concentration of TW20 is close to 0.006 whereas with increase in TW20 concentration it reaches to a value of 0.09 (figure S3) which indicates that fisetin molecules present in a rigid environment where the rotational motion is restricted, as process of micellization increases.

Fluorescence Study of Fisetin in TW20:cholesterol(1:1) Niosome Membrane

Figure 2 shows the emission behaviour of fisetin in aqueous buffer (pH 6.8), TW20 micelle and TW20:cholesterol(1:1) niosome membrane. It is observed that in buffer solution, fisetin possesses a broad structureless emission band at ~ 498 nm whereas in TW20 micelle and niosome membrane, the bands are structured and distinct at ~ 410 nm, 473 nm and 535 nm respectively. These emission wavelengths are attributed to neutral,

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excited state anion and tautomeric emission of fisetin as observed in many organized media like bile salt, DNA medium and lipid bilayer membrane [4,5,11]. The presences of these bands confirm that both TW20 micellar medium and niosome membrane are suitable candidate to observe the ESIPT behaviour of fisetin. The variation in emission maxima of different prototropic emission of fisetin in different media (water, TW20 micelle, TW20:cholesterol(1:1) niosome membrane) are clearly visualized from the inset of figure 2. The emission spectrum of fisetin is significantly modulated by temperature in TW20:cholesterol(1:1) niosomes such as: at lower temperature (10 ^{o}C) all the three bands are distinct whereas at higher temperature (60 ^{o}C) the tautomeric peak becomes structureless. The reason behind this significant decrease is discussed later.

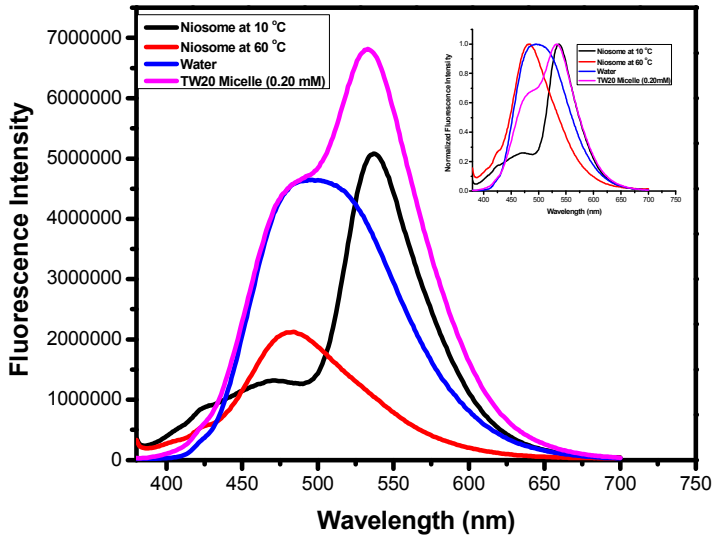

Figure 2: Plot for emission of fisetin in buffer, TW20 micelle and TW20:cholesterol(1:1) niosome membrane, inset shows the normalized fluorescence intensity of fisetin in water, TW20 micelle and in TW20:cholesterol(1:1) niosome membrane (at 10 and 60 ^{o}C).Variation in different emission bands indicate the efficiency of ESIPT process of fisetin in different media. [Fisetin] = 5μM, λ_{ex} = 370 nm, slit width = 5/5 nm.

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Partition Coefficient Calculation

Partition coefficient (K_p) of a fluorescent molecule in an organized media can be calculated by using fluorescence intensity of the partitioning molecule [30, 31]. In the present study, the fluorescence intensity of fisetin tautomeric emission (535 nm) is used to estimate the partition coefficient of fisetin in TW20:cholesterol(1:1) niosome membrane using equation (6),[32] where Y = fluorescence intensity of fisetin in TW20:cholesterol(1:1) niosome membrane, Y_{max} = maximum fluorescence intensity of fisetin in TW20:cholesterol(1:1) niosome membrane, K_d = dissociation constant. The reciprocal of K_d gives the value of partition coefficient. The simplified form of equation (6) is represented in scheme S1.

$$Y = Y_{max} \frac{[Fisetin]}{K_d + [Fisetin]} \quad \text{..... (6)}$$

Figure 3A represents the emission behaviour of fisetin in TW20:cholesterol(1:1) niosome membrane with variation in fisetin concentration and figure 3B represents the double reciprocal plot of $\frac{1}{Y}$ against $\frac{1}{Y_{max}[Fisetin]}$. The inset of figure 3A represents the point plot of the emission intensity at 535 nm with variation in fisetin concentration. This double – reciprocal plot of $\frac{1}{Y}$ against $\frac{1}{Y_{max}[Fisetin]}$ is linear and K_d is estimated from the slope of the plot. The partition coefficient value of fisetin in TW20:cholesterol(1:1) niosome membrane at lower temperature (10 ^oC) is found to be 2.7×10^4. At higher temperature (60 ^oC), the anionic and tautomeric emission band of fisetin are overlapped to give a broad emission band (figure S6) so it is not convenient to calculate the partition coefficient value at that temperature.

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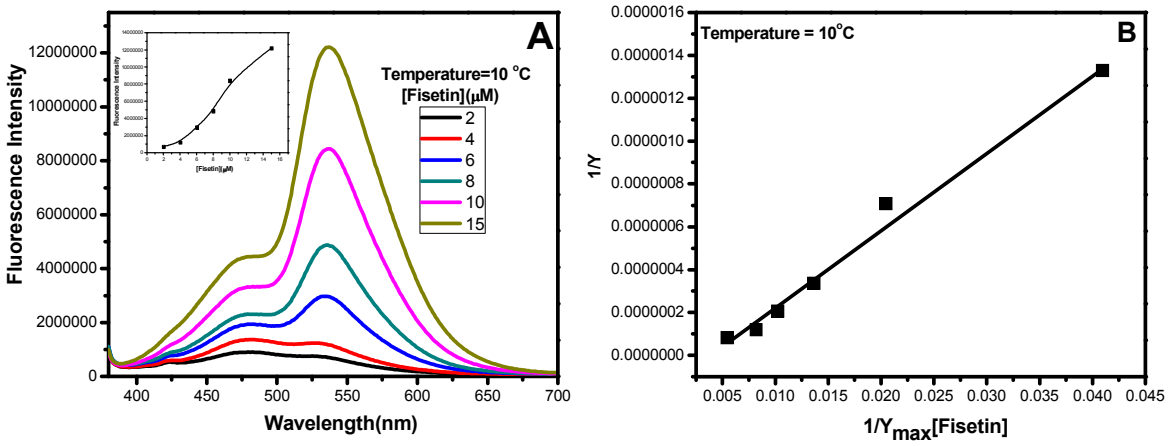

Figure 3: (A) Emission spectra of fisetin in TW20:cholesterol(1:1) niosome membrane with increase in fisetin concentration at lower temperature(10 °C), the inset shows the point plot for the emission of fisetin phototautomer emission (535 nm) in TW20:cholesterol(1:1) niosome membrane with increase in concentration of fisetin, The saturation limit in the point plot indicates the partitioning behaviour of fisetin in niosome membrane (B) Double reciprocal plot of $\frac{1}{Y}$ against $\frac{1}{Y_{max}[Fisetin]}$ at 10 °C. λ_{ex} = 370 *nm*, λ_{em}= 535 *nm*.

Temperature Dependent Fluorescence Intensity Study

Observations

Figure 4 shows the emission behaviour of fisetin in TW20:cholesterol(1:1) niosome membrane as a function of temperature. The interesting observations are as follow : (i) two iso-emissive points are observed at ~ 451 nm and ~ 507 nm respectively, (ii) fluorescence intensity of tautomeric and neutral emission decrease with a concomitant increase in anionic emission, (iii) position of emission maximum of tautomeric (535 nm)

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and neutral emission (430 nm) remain intact whereas there is a red shift in anionic emission maximum (470 - 481 nm) with temperature (figure 3A), (iv) the intensity ratio $I_{tautomer}/I_{anion}$ (I_T/I_A) value decreases with increase in temperature (figure 3B).

Discussions

The first observation of figure 3A says about the presence of two iso-emissive points, which confirm that there are two equilibria exist between three emitting species of fisetin in TW20:cholesterol(1:1) niosome membrane (neutral emission, anionic emission and tautomeric emission). Presences of three emitting species indicate that niosome membrane is favorable for the ESIPT process of fisetin. Fluorescence intensity of neutral (430 nm) and tautomeric emission (535 nm) decrease with a concomitant increase in anionic emission (470 nm) (figure 4A, figure S7 A). The possible explanation for this variation in intensities may be due to the wetting in the interfacial domain of niosomal membrane with increase in temperature, as reported in our previous study [17]. With increase in temperature, the niosomal interfacial domain gets hydrated which results in the retardation of ESIPT process. This result in decrease in the fluorescence intensity of fisetin tautomeric emission in contrast, increases the fluorescence intensity of anionic emission. The third observation gives information about the polarity of the surrounding environment of fisetin in niosome membrane with temperature. Out of the three emitting species, only the anionic species show solvatochromism through the shift of its emission maximum by ~11 nm towards longer wavelength region (470-481 nm) whereas the positions of neutral and tautomeric emission maximum remain unchanged. Shifting towards red region indicates that the polarity of the surrounding environment of fisetin anion increases with temperature (figure S7 B). The intensity ratio plot $I_{tautomer}/I_{anion}$ decreases with temperature (figure 4B). It is

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reported that the tautomeric emission of fisetin originates from relatively hydrophobic environment as compare to anionic emission [5]. As this ratio decreases it indicates that the hydrophobicity of the niosome membrane decreases with increase in temperature. The accessibility of water increases with increase in temperature which is responsible for the decrease in medium hydrophobicity.

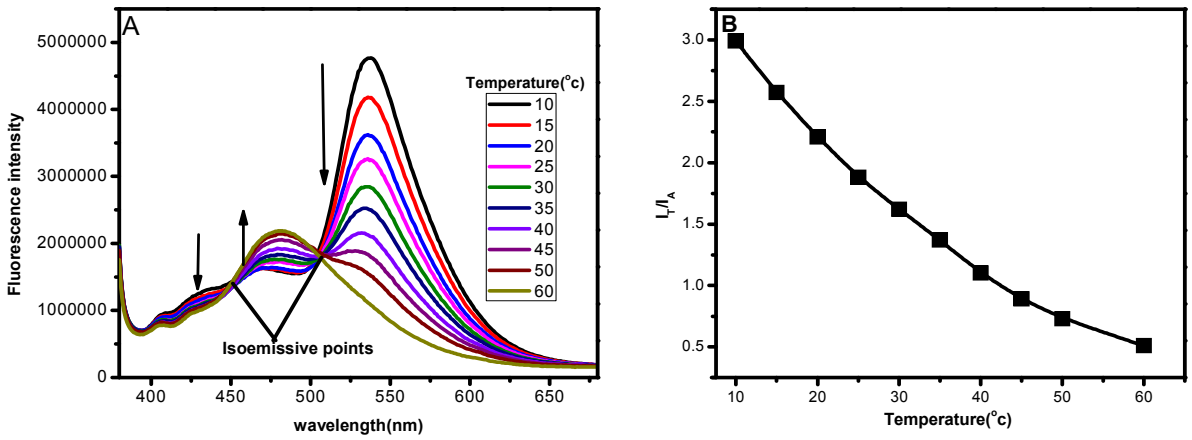

Figure 4: (A) Emission spectra of fisetin in TW20:cholesterol(1:1) niosome membrane with variation in temperature. The variations in the fluorescence intensity of different prototropic emissions of fisetin indicate the temperature induced changes of niosomal membrane (B) Plot for the fluorescence Intensity ratio of (I_T/I_A) of fisetin in TW20:cholesterol(1:1) niosome membrane with variation in temperature. The decrease in intensity ratio (I_T/I_A) with variation in temperature indicates the change in hydrophobicity of the surrounding environment of fisetin in TW20:cholesterol(1:1) niosome membrane. [Fisetin] = 5 μM, λ_{ex} = 370 nm, slit width = 5/5 nm.

Figure 5A represents the emission spectra (λ_{ex} = 418 nm, λ_{em} = 490 nm) and figure 5B represents the excitation spectra (λ_{em} = 535 nm) of fisetin in TW20:cholesterol(1:1)

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niosome membrane as a function of temperature. The emission spectra at λ_{ex} = 418 nm increases with increase in temperature. The excitation spectra at λ_{em} = 535 nm gives two peaks at ~ 370 nm and ~ 418 nm respectively. Presence of an iso-emissive point at ~ 404 nm indicates that at ground state two different species of fisetin exist. With increase in temperature the formation of ground state anionic species increase which is confirmed from both emission (figure 5A) and excitation spectra (figure 5B). Selvam et.al have reported that there are two reasons for ground state anion formation i.e. (i) if there is a proton acceptor in the surrounding environment of fisetin in the ground state and (ii) if the medium has in alkaline pH. For this study phosphate buffer solution having pH 6.8 and strength 1 mM was used so the possibility of medium alkalinity for ground state anion formation is discarded. The main contributing factor for the ground state anion formation is the presence of proton acceptor in the fisetin environment. It is expected that the OH group of TW20 and the negative charge of monosodium phosphate and disodium phosphate (phosphate buffer solution) are responsible for the formation of ground state anion of fisetin in TW20:cholesterol(1:1) niosome membrane.

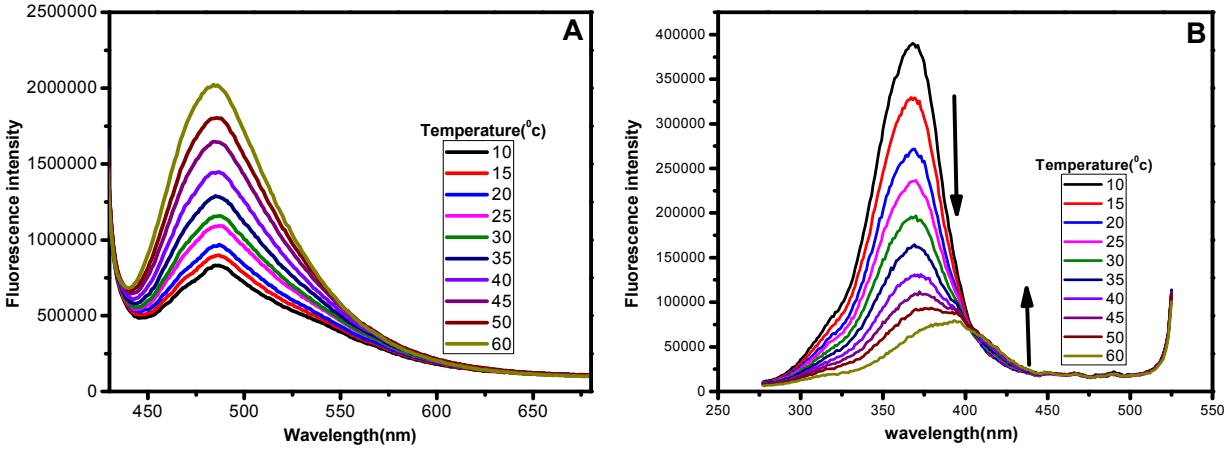

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Figure 5: (A) Emission spectra of fisetin in TW20:cholesterol(1:1) niosome membrane with variation in temperature (λ_{ex} = 418 nm, λ_{em} = 490 nm). The increase in fluorescence intensity of fisetin upon excitation at 418 nm with increase in temperature indicates the formation of ground state anion in TW20:cholesterol(1:1) niosome membrane. (B) Excitation spectra of fisetin in TW20:cholesterol(1:1) niosome membrane with variation in temperature (λ_{em} = 535 nm), presence of second peak at 418 nm (figure 5B) confirms the formation of ground state anion in TW20:cholesterol(1:1) niosome membrane. [Fisetin] = 5 μM, slit width = 5 nm.

The existence of multipleprototropism of fisetin in TW20:cholesterol(1:1) niosome membrane is confirmed through fluorescence intensity study(figure 4, figure 5). Phosphate buffer (monosodium phosphate and disodium phosphate) solution having pH 6.8 and strength 1 mM was used for the preparation of niosome solution. So in order to get a clear idea about the role of surface charge on the multipleprototropism of fisetin, we have measured zeta potential of niosomal solution in the presence and absence of fisetin (figure S8).The constant value of zeta potential implies that there is no role of surface charge on the multiple prototropism of fisetin.

Location Study

Cetylpyridinium chloride (CPC) is a well known interfacial quencher for fluorophore resides on the interface of the lipid bilayer membrane [11, 31, 33]. Due to the presence of a positive charge on the head group region of CPC it prefers to stay near the negatively charged phosphate group of the phospholipid molecule in the interface of bilayer membrane. CPC quenches the fluorescence intensity originates from interfacial region.

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Mohapatra *et.al*[11]and Swain *et.al* [31] have reported the interfacial location of fisetin and capsaicin in lipid bilayer membrane by using CPC induced quenching of both fisetin and capsaicin respectively. As niosomes are structurally similar to liposomes, it is expected that CPC quenching study will identify the location of fisetin in niosome membrane also.

Figure 6 shows the CPC induced quenching of fisetin in TW20:cholesterol(1:1) niosome membrane. The maximum concentration of CPC used was 0.08 mM which is well below the CMC of CPC (0.98 mM). As the concentration of CPC is very less, there is no possibility of micelle formation. It is observed that, there is a decrease in both anionic and tautomeric emission of fisetin in TW20:cholesterol(1:1) niosome membrane with increase in CPC concentration at both lower (10 ^{o}C) and higher (50 ^{o}C) temperatures (figure 6A, figure S9). The detectable observation at higher temperature (50 ^{o}C) is that, the separation between the emission maximum of both anionic and tautomeric emission decrease as compare to lower temperature (figure S9). To avoid the interference of spectral contribution, it is not convenient to do the location study at higher temperature (50 ^{o}C) (figure S9). The tautomeric emission is more intense then anionic emission and it mostly originates from water deficient environment so to get a clear idea about the location of fisetin tautomeric species here location study is carried out by considering the fluorescence intensity and fluorescence lifetime of FT* emission. The Stern-Volmer plot (F_o/F versus [CPC]) for the quenching of fisetin fluorescence is given by figure 6B. The value of Stern-Volmer quenching constant (K_{sv}) was calculated from the slope of the equation (7) [19].

$$\frac{F_0}{F} = 1 + k_{sv}[Q] \quad \text{..... (7)}$$

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The value of K_{sv} was found to be 5.9×10^3 M^{-1} .The higher value of K_{sv} and constant lifetime value indicates the occurrence of static quenching. Thus CPC quenching study confirms that the tautomeric species of fisetin originate from interfacial domain of niosomal membrane whereas it is expected that the anionic species are distributed both in interface and bulk water region.

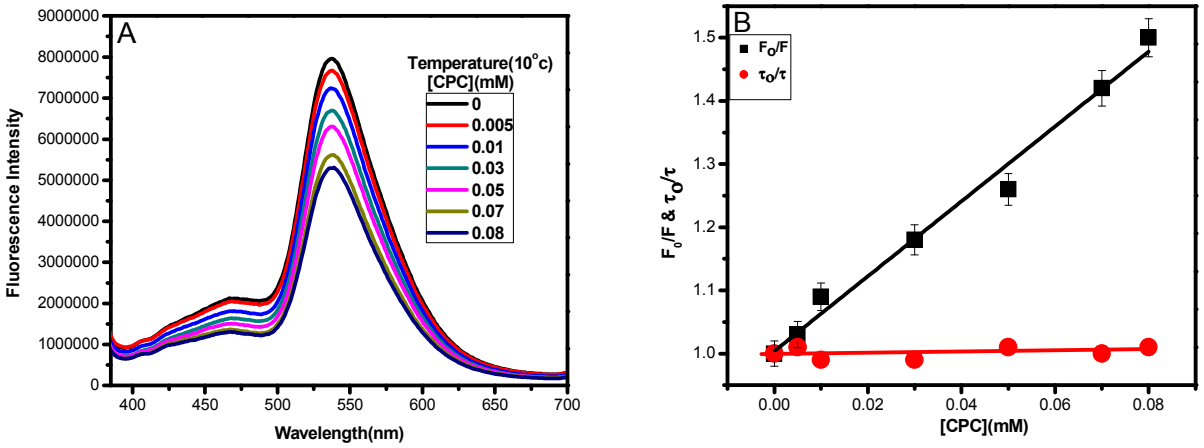

Figure 6: (A) Emission spectra of fisetin (λ_{ex} = 370 nm) in TW20:cholesterol(1:1) niosome membrane with varying concentration of CPC at 10 ^oC, decrease in both anionic and tautomeric emission intensity indicate the interfacial location (B) Stern-Volmer plot (F_o/F) versus [CPC] and τ_o/τ plot for the quenching of phototautomer form of fisetin in TW20:cholesterol(1:1) niosome membrane by CPC at 10 ^oC. Constant value of τ_o/τ represents that CPC induced quenching is static in nature. [Fisetin] = 5 μM, slit width = 5/5 nm, Error = ± 3%.

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Fluorescence Anisotropy Study

Fluorescence anisotropy is an efficient spectroscopic parameter to estimate the rigidity of an organized media by using a fluorescent molecular probe [19]. The fundamental anisotropy of fisetin tautomeric emission in glycerol medium at -10 ^{o}C is reported as 0.36 [11]. Whereas in homogeneous THF medium r_{ss} value is reported as 0.06 at 25 ^{o}C. In liposome medium the anisotropy values are 0.27 and 0.22 at 15 ^{o}C and 35 ^{o}C respectively [11]. In TW20:cholesterol(1:1) niosome membrane, anisotropy of tautomeric species decrease with increase in temperature. The anisotropy value of tautomeric species decrease from 0.13 to ~ 0.04 as a function of temperature. This decrease value of anisotropy indicates that fisetin tautomeric species are located in a more restrict environment at lower temperature whereas the surrounding environment becomes mobile with increase in temperature. Anisotropy value indicates that the rigidity in the surrounding environment of fisetin in the liposome membrane is more than that of TW20:cholesterol(1:1) niosome membrane. The Microviscosity value gives information about the resistance offered by a single molecule in an organized environment [23, 25]. For this study microviscosity is calculated by using equations 3, 4. Like anisotropy, microviscosity also decreases with increase in temperature. As location study confirms that, the tautomeric emission originates from interfacial domain of TW20:cholesterol(1:1) niosome membrane. Our previous study reported that, there is a wetting in niosomal interfacial domain with increase in temperature [17], so this decrease in anisotropy can be rationalized by using the wetting concept. The interfacial wetting in niosomal membrane increases the rotational motion of fisetin tautomeric species which results in decrease the anisotropy value. As fluidity of the membrane increases the microviscosity decreases and polarity increases.

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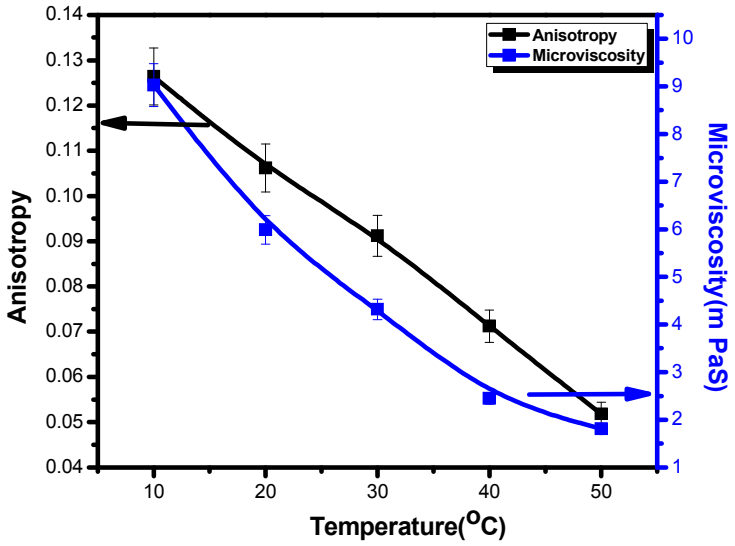

Figure 7: Plot of variation of fluorescence anisotropy and microviscosity (mPa S) of tautomeric emission of fisetin in TW20:cholesterol(1:1) niosome membrane. Decrease in anisotropy and microviscosity values indicate the change in rigidity at the niosomal interfacial domain with variation in temperature. [Fisetin] = 5 μM, λ_{ex} = 370 nm, λ_{em} = 535 nm, Error = ± 4%.

Fluorescence Lifetime Study

Fluorescence lifetime decay of both anionic and tautomeric emission of fisetin follow biexponential fitting in TW20 micelle (figure S4, S5). For both the species there is no change is lifetime value throughout the TW20 concentration (Table S1, Table S2). Not only lifetime value there is no change in relative amplitude and population also (Table S1, Table S2). In TW20:cholesterol(1:1) niosome membrane also the FT* emission shows biexponential fitting (figure S10). The fluorescence lifetime value and the decay profile of fisetin in TW20:cholesterol(1:1) niosome membrane are represented in figure S10 and table 1 respectively. For similar observation in DMPC lipid bilayer membrane, Mohapatra

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et al. have reported that there is a local heterogeneity in the distribution of phototautomer, though CPC quenching study established its location mostly at the interfacial region [11]. They reported that, due to the presence of different modes of interaction of fisetin hydroxyl groups with the ester linkages at the interfacial domain, the local heterogeneity is established. Like liposomes in TW20:cholesterol(1:1) niosomes also fisetin tautomeric species follow biexponential fitting having a shorter lifetime value of ~2 ns and a longer lifetime value of ~ 4 ns. The average lifetime value along with both the shorter and longer components decrease with increase in temperature. The measured drop in fluorescence lifetime in ~ 43% as the niosomal interfacial hydration increases with increase in temperature. This dropping in fluorescence lifetime is also agree with the dropping in fluorescence intensity of FT* emission. This indicates that with increase in temperature the nonradiative decay rates increase which is responsible for this decrease. Another interesting observation is that, the relative amplitude of shorter lifetime component increases along with a decrease in the relative amplitude of longer component. In our previous study [18] we have reported that in polar aprotic solvent (Acetonitrile), FT* emission of fisetin has an emission maximum at 535 nm and has a lifetime value of ~ 0.7 ns whereas in ethanol the FT* emission follows biexponential fitting having a shorter (~ 0.4 ns) and a longer (~ 3.02 ns) lifetime component. The longer component can be attributed to the hydrogen bonded stabilized species of fisetin as the polar protic solvent has an ability to form the hydrogen bond with probe molecules. So, it is expected that the longer lifetime component originates from interfacial region accessible towards water whereas the shorter component originates from the inner site of the interface. The changes in relative amplitudes of both the lifetime components of the phototautomer form with

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increase in temperature suggest that fisetin molecules get redistributed with increase in population in the inner site of the interfacial domain at the expense of population in the water accessible interfacial site.

Figure 8 represents the lifetime distribution plot of FT* emission in TW20:cholesterol(1:1) niosome membrane at 10 and 50 ^{o}C. It is observed that the full width at half maximum (FWHM) value of shorter component remains constant whereas the FWHM value of longer component decreases with increase in temperature (table 2).The modal lifetime values of both shorter and longer component decrease with increase in temperature. As it is explained that the shorter lifetime component originates from water deficient inner site of the niosomal membrane whereas the longer component originates from the water accessible outer site of the niosomal membrane, the constant value of FWHM for shorter component both at 10 and 50 ^{o}C indicate that the local heterogeneity sense by FT* emission is same whereas the heterogeneity sense by longer component is more at lower temperature(10 ^{o}C) and it approaches homogeneity at higher temperature(50 ^{o}C).

Table 1: Temperature dependent fluorescence lifetime value (nanosecond) of FT* emission in TW20:cholesterol(1:1) niosome membrane. λ_{ex} = 370 nm, λ_{em} = 535 nm, [Fisetin] = 5 μM. Error = ± 5%

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Temp(°C)	τ_1,α_1	τ_2,α_2	τ_{avg}	$\chi 2$
10	$1.9_0(61)$	$4.0_5(39)$	3.14	1.04
20	$1.6_1(76)$	$3.9_0(24)$	2.60	1.07
30	$1.2_4(80)$	$3.6_2(20)$	2.24	1.16
40	$0.9_8(81)$	$3.4_8(19)$	2.11	1.15
50	$0.8_0(85)$	$3.1_8(15)$	1.78	1.18

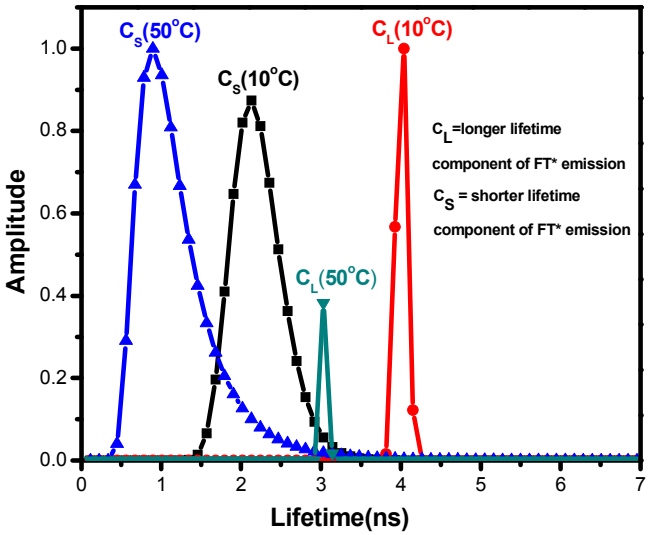

Figure 8: Fluorescence lifetime distribution plot for both longer and shorter components of FT* emission in TW20:cholesterol(1:1) niosome membrane at 10 and 50 ^{o}C, wider distribution indicates that local heterogeneity sense by fisetin is more whereas narrower distribution indicates the homogeneity of the local environment, [Fisetin] = 5 μM, λ_{ex} = 370 nm, λ_{em} = 535 nm.

Table 2: The fluorescence lifetime distribution data of FT* emission in TW20: cholesterol (1:1) niosome membrane at 10 and 50 ^{o}C. [Fisetin] = 5 μM. λ_{ex} = 370 nm, λ_{em} = 535 nm, (Error = ± 5 %).($FWHM_S$ = Full width at half maximum of shorter component, $FWHM_L$ =

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Full width at half maximum of longer component), both modal lifetime and FWHM in nanosecond.

Temp(°C)	FWHMs	$FWHM_L$	Modal time $_S$	Modal time $_L$	$\chi 2$
10	0.7	0.18	2.1_3	4.0_3	1.15
60	0.7	0.11	0.8_9	3.0_2	1.01

Fluorescence Anisotropy Decay Study

The fluorescence anisotropy decay and the corresponding rotational parameters of fisetin phototautomeric species (FT*) in TW20:cholesterol(1:1) niosome membrane are given in figure 9 and table 3 respectively. It is observed that the anisotropy decay of FT* emission follows biexponential fitting having rotational time constants (τ_1(r)) 1.04 ns and (τ_2(r)) 16.50 ns respectively (table 3) with an average rotational time of 15.57 ns. In methanol fisetin phototautomeric species follow monoexponential fitting having a rotational time (τ_1(r)) of 0.21 ns [5]. The longer rotational time constants in TW20:cholesterol(1:1) niosome membrane as compare to methanol indicate that the resistance offered by niosomal membrane to a small molecule is greater than that of homogeneous solvent. Like TW20:Cholesterol(1:1) niosome membrane the TritonX:Cholesterol niosome membrane is also offered longer rotational time constants(~20 ns) to small molecules as reported by Paul et.al [34]. From location and lifetime studies it is observed that the phototautomeric species are redistributed in the water deficient inner site and water accessible outer site of the niosomal interfacial domain so it can be concluded that the rotational motion of the tautomeric species present in the water accessible region result in the origination of 1.04 ns rotational time whereas the longer rotational time of 16.50 ns originates because of the rotation of fisetin tautomeric species which are distributed in the inner site of the niosomal

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interfacial domain (more restricted environment). To get a better understanding about the motion of the fisetin phototautomeric species in TW20:cholesterol(1:1) niosome membrane we have calculated the order parameter S by using the equation S = $\sqrt{a_{2r}}$ (here a_{2r} represents the relative amplitude of slower rotational time) [20].The value of S varies from 0 (unrestricted motion) to 1(completely restricted motion). In this case value of S is found to be 0.88 which indicates that fisetin tautomeric species reside on the restricted environment in TW20:cholesterol(1:1) niosome membrane.

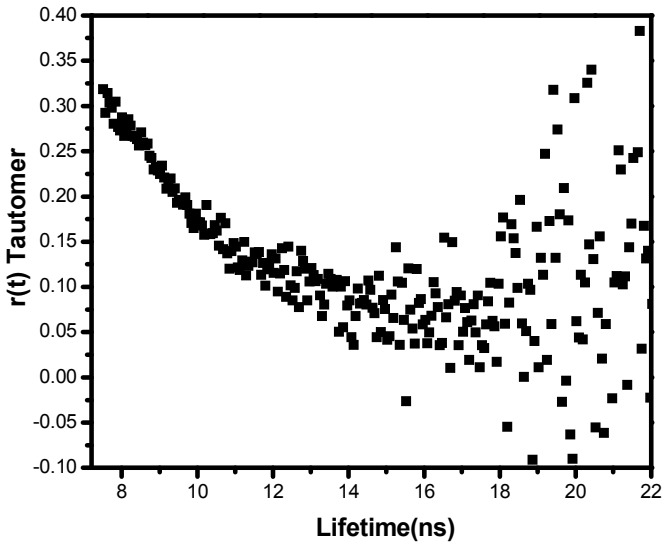

Figure 9: Fluorescence anisotropy decay of fisetin in TW20:cholesterol(1:1) niosome membrane at room temperature.[Fisetin] = 5 μM, λ_{ex} = 370 nm,λ_{em} = 535 nm, error = ± 5%

Table 3: Rotational dynamics parameters of fisetin phototautomer (FT*) species in TW20:cholesterol(1:1) niosome membrane. [Fisetin] = 5 μM, λ_{ex} = 370 nm,λ_{em} = 535 nm, rotational time(τ_r) is in nanosecond (ns),error = ± 5%, $\tau_{avg} = \tau_{1r}*a_{1r} + \tau_{2r}*a_{2r}$

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τ_{1r}, a_{1r}	τ_{2r}, a_{2r}	τ_{avg}	r_0	χ^2
1.04(6)	16.50(94)	15.57	0.33	1.24

Scavenging Activity of Fisetin in Niosome Membrane using DPPH radical

Figure 10 represents the DPPH-scavenging activity of fisetin in TW20:cholesterol(1:1) niosome membrane as a function of time. The absorption spectra and corresponding point plot for the absorbance value of DPPH radical (at 524 nm) in fisetin loaded niosome membrane is represented in figures S11 and S12 respectively. The absorption maximum of DPPH at 524 nm is well known in 1:1 mixture of ethanol and buffer [35]. In TW20:cholesterol(1:1) niosome membrane also DPPH radical has an absorbance maximum at 524 nm (figure S11). The antioxidant activity of fisetin as a function of time is calculated by using equation 5. It is observed that with increase in time the scavenging activity of fisetin loaded in niosome membrane increases. This increase value of scavenging activity indicates that fisetin may be used as a good antioxidant in TW20:cholesterol(1:1) niosome membrane.

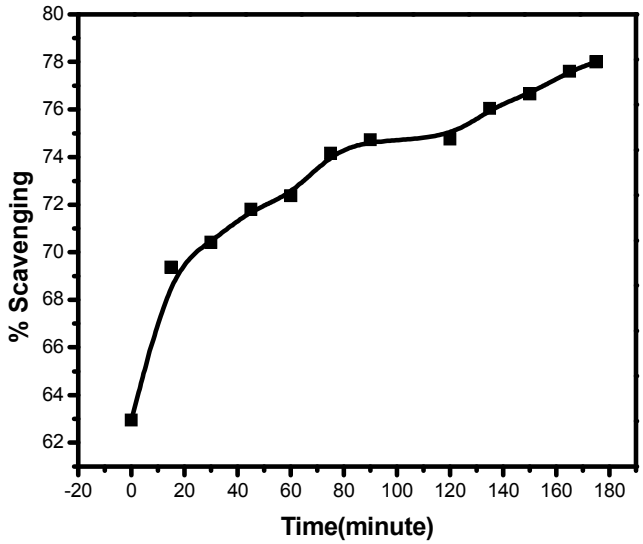

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Figure 10: DPPH Scavenging activity of fisetin in TW20:cholesterol(1:1) niosome membrane with variation in time. The scavenging activity increases with increase in time. [Fisetin] = 5 μM, [DPPH] = 50 μM.

Conclusions

This study use the multiple fluorescence parameters (intensity, transition energy, fluorescence lifetime, fluorescence anisotropy) of the four prototropic forms (ground state anion, excited state anion, neutral form emission and phototautomer emission) of fisetin to understand the TW20:cholesterol (1:1) niosomal membrane and its changes in hydration level with temperature. There is no role of surface charge on the multiprototropic emission of fisetin as observed from zeta potential measurement. Fisetin partitions well into the TW20:cholesterol(1:1) niosome membrane having partition coefficient value 2.7×10^4 M^{-1} at 10 ^oC. The bathochromic shift of anionic emission (470 - 481 nm) gives information about the water accessibility at the niosomal interfacial domain with variation in temperature. The motional resistance offered by the interfacial domain of the niosomal membrane to small molecules was estimated by calculating microviscosity using fluorescence anisotropy and fluorescence lifetime values. The microviscosity value at the niosomal interfacial domain decreases from 9 mPa S to 1.81 mPa S with increase in temperature. Fisetin phototautomeric species are observed to get distributed in the two different regions of niosomal interfacial domains (the water-accessible outer side and the water deficient inner side) having two different rotational time constants as observed from fluorescence anisotropy decay study. The interfacial location of fisetin in

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TW20:cholesterol(1:1) niosome membrane could have an important implication with regards to antioxidant activity as confirmed from DPPH scavenging study.

Acknowledgements:

This research was supported by a research project funded by the Department of Science and Technology, Government of India. The authors thank Dr. E. Prasad, Department of chemistry, IIT madras, for his kind help in carrying out DLS experiments. J.M thanks IIT madras and J.S thanks DST for fellowship.

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Location of fisetin molecules in Niosomal membrane