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PAPER

Multiple prototropism of fisetin in sodium cholate and related bile salt media†

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Fisetin, a bioflavonoid, has important biological relevance. It exhibits *intramolecular excited state proton transfer* (ESIPT), analogous to the structurally similar flavonoids. The presence of multiple prototropic forms of fisetin was observed at various concentrations of different bile salt molecules. The presence of ground state fisetin anion $(FA)_{GS}$ (λ_{ex} 418 nm; λ_{em} 490 nm) in alcohols and bile salt micellar media is a novel observation. The interaction of fisetin with sodium cholate (NaC) and some other bile salts has been studied in detail, using the intrinsic fluorescence of different prototropic forms of fisetin: neutral form (FN, λ_{ex} 369 nm, $\lambda_{em} \sim$ 400 nm), ground state anion form (($FA)_{GS}$, λ_{ex} 418 nm, λ_{em} 490 nm) and phototautomer (FT, λ_{ex} 369 nm, λ_{em} 540 nm). The hypsochromic shift of $(FA^*)_{ES}$ emission and bathochromic shift of FT emission with increasing bile salt concentration suggests the progressive reduction of polarity of the bile salt media, which could be resulting from the neutralization of bile salt molecules as their concentration increases.
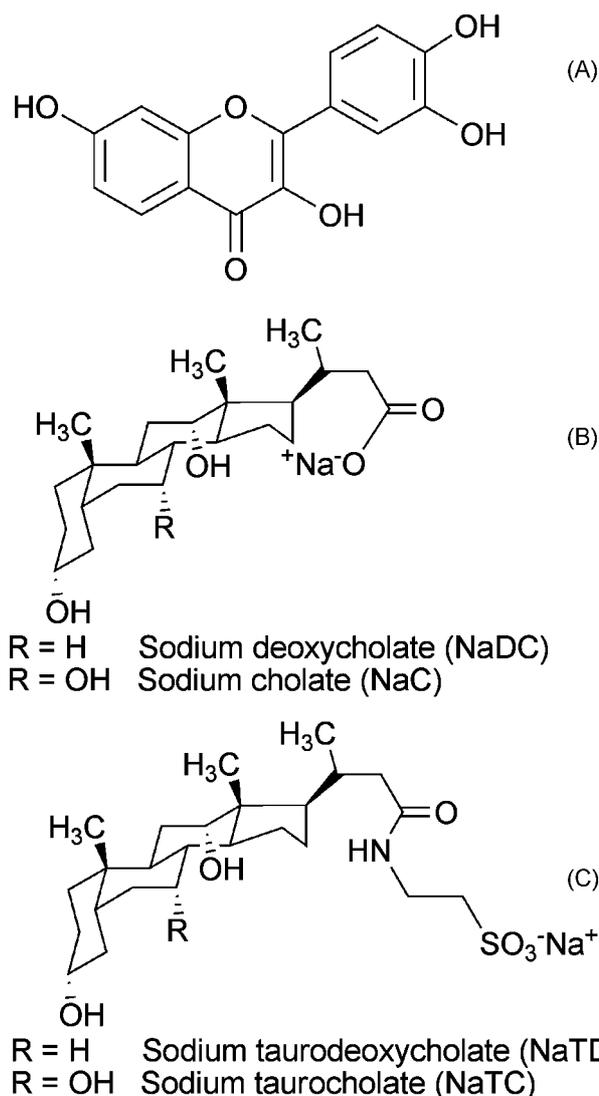
Introduction

Fisetin (Chart 1A) belongs to a class of bioflavonoids, which are polyphenolic compounds present in common plant-based food items, such as onions, apples, tea and beverages, like red wine.[1–3] The biological activities of fisetin are various. It has antioxidant property (analogous to vitamin E), inhibits protein kinase C, is used in the treatment of AIDS, anti-inflammatory activity in brain microglia, enhances long-term memory and it is present in dietary supplements.[4] The optical absorption and fluorescence properties of fisetin are quite interesting and challenging. It exhibits *intramolecular excited state proton transfer* (ESIPT) and shows two emission peaks in organized media like liposome and cyclodextrins; one peak is at 400 nm due to $S_1 \rightarrow S_0$ emission and the other peak at a red-shifted wavelength, 540 nm due to phototautomerization of the neutral molecule under excitation.[5–11] Recently the presence of anionic species of fisetin was observed in alkaline pH.[6]

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† Electronic supplementary information (ESI) available: Fig. S1 (absorption spectra of fisetin in different solvents); Fig. S2 (fluorescence spectra of fisetin in different solvents); Fig. S3 (excitation spectra of fisetin in different NaC concentrations); Fig. S4 (deconvoluted emission spectra of FN (λ_{ex} 369 nm) in buffer and NaC); Fig. S5 (EEMF spectra of fisetin in buffer medium and NaC at 25 °C); Fig. S6 (absorption spectra of fisetin in different bile salts); Fig. S7 (emission spectra of FT and $(FA)_{GS}$ in different bile salts); Fig. S8 (plot of fluorescence intensity of FT emission and $(FA)_{GS}$ emission against different bile salt concentrations at various T); Fig. S9 (plot of fluorescence anisotropy of FT emission and $(FA)_{GS}$ emission against different bile salt concentrations at various T) and Fig. S10 (EEMF spectra of fisetin in buffer medium and NaC at 45 °C). See DOI: 10.1039/c0pp00120a

Bile salts are biologically important molecules, synthesized from cholesterol within the liver. Bile salts have a hydrophobic steroidal backbone with one to three hydroxyl groups and a carboxyl side chain lying along in the same plane of hydroxyl groups (Chart 1B and Chart 1C). This structure leads to a unique aggregation pattern, accounting for their solubilization of both hydrophobic and hydrophilic solutes.[12,13] Consequently bile salts have received much attention as drug delivery media.[13] The micellization of bile salts is a multi-step process; initially dimeric primary aggregates are formed by hydrophobic interaction between steroidal domains and as the concentration of these bile salts is increased they lead to the formation of larger secondary aggregates, which are formed primarily due to the hydrogen bonding between hydroxyl and carboxyl groups of different dimeric bile salt units.[14–16] The secondary bile salts are derived from the primary bile salts (cholates and chenodeoxycholates) and conjugated to either taurine or glycine amino acids through a peptide linkage.[12] For the present work, four bile salts, namely sodium cholate (NaC), sodium deoxycholate (NaDC), sodium taurocholate (NaTC) and sodium taurodeoxychoalte (NaTDC), are used. Among these four bile salts, NaC and NaDC are primary (unconjugated) bile salts, whereas NaTC and NaTDC are secondary (conjugated) bile salts. The bile salt aggregates are considered as pseudo-micellar species, since they do not have a sharp monomer to aggregate transition, which is characterized by well defined critical micellar concentrations (cmc). Instead they have a cmc range, *viz.* for NaC and NaTC of 12–16 mM, and for NaDC and NaTDC of 4–6 mM.[16]

The absorption and fluorescence properties of fisetin show a simultaneous presence of more than one prototropic equilibria in different media,[5,6] which implies simultaneous presence of more than two prototropic forms. This phenomenon has been referred to as 'multiple prototropism' in the present discussions.

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Chart 1 Chemical structure of (A) 3,4,3′,4′-tetrahydroxyflavone (fisetin), (B) and (C) bile salts.

An understanding of the interaction between bile salts (anionic surfactants) and fisetin, exhibiting various prototropic forms is important. The study will help in establishing bile salts as possible drug delivery media for other pharmaceutically important flavonoids. The objective of this work is to study the interaction between fisetin and all the four bile salts by analyzing the intrinsic photophysical properties of different prototropic forms of fisetin.

Experimental

Materials

Fisetin, sodium taurodeoxycholate (NaTDC) and sodium taurocholate (NaTC) were purchased from Sigma Chemical Company (USA) and were used without any further purification. Sodium deoxycholate (NaDC) and sodium cholate (NaC) were purchased from S. D. Fine Chemicals, India. Spectroscopy grade solvents were obtained from Sisco Research Laboratories, India.

Analytical grade ethanol was obtained from Merck, USA. The stock solutions of fisetin for the homogeneous media study were prepared by dissolving it in the required solvents separately.

Measurements

The absorption spectra were recorded using Jasco –V550 UV-Visible spectrophotometer and fluorescence spectra using Hitachi F4500 model spectrofluorimeter. The excitation and emission slits were set to a bandwidth of 10 nm. Temperature control was attained by using a double walled cuvette holder; connected to an INSREF thermostat with an accuracy of ±0.1 °C. For steady-state fluorescence anisotropy measurements, polacoat grating polarisers and Glan-Thompson polarisers were used. The steady state fluorescence anisotropy is defined as:[17]

$$r_{ss} = \frac{I_{VV} - G I_{VH}}{I_{VV} + 2 G I_{VH}}$$

where, I_{VV} and I_{VH} are the fluorescence intensities and the subscript indicates the vertical (V) and horizontal (H) orientations of the excitation and emission polarizer. G is the instrumental correction factor,

$$G = \frac{I_{HV}}{I_{HH}}$$

The fluorescence decays are collected using Horiba Jobin Yvon TCSPC Lifetime system with FluoroHub single-photon counting controller module and equipped with the TBX single-photon detection module, typically of 180 ps FWHM. The NanoLED-03 nanosecond source with UV output at 370 nm, typical optical pulse duration of 1.3 ns FWHM and a repetition rate of 1 MHz was used. The decay curves are further analysed using the DAS6 analysis software. A fitted value with $0.99 \leq \chi^2 \leq 1.2$ was accepted. The emission spectra of fisetin in different bile salts are deconvoluted using a multi-peak fitting of Gaussian spectrum in Origin 6.0 software and the correlation factor (r) is maintained at ~1.0

Preparation of fisetin–bile salt solutions

Fisetin was first dissolved in ethanol and further diluted with bile salt solutions having concentrations above their cmc (20.0 mM for NaDC and NaTDC; 48.0 mM for NaC and NaTC). In these solutions, the final fisetin concentration is maintained constant at 10 μM and varying bile salts concentrations were prepared by appropriate addition of bile salt solution and the concentration of ethanol was kept at 2%. To maintain the physiological condition, pH was kept constant at 7.4 with 50 mM sodium phosphate buffer for all bile salt experiments.

Results and discussion

Photophysical behavior of fisetin in sodium cholate aggregates

Absorption and emission spectral features. The absorption and fluorescence spectral behavior of fisetin in NaC aggregates is given in Fig. 1.

Fig. 1A shows an intense absorption peak at 369 nm and a slightly enhanced absorption at 418 nm. The peak at 369 nm
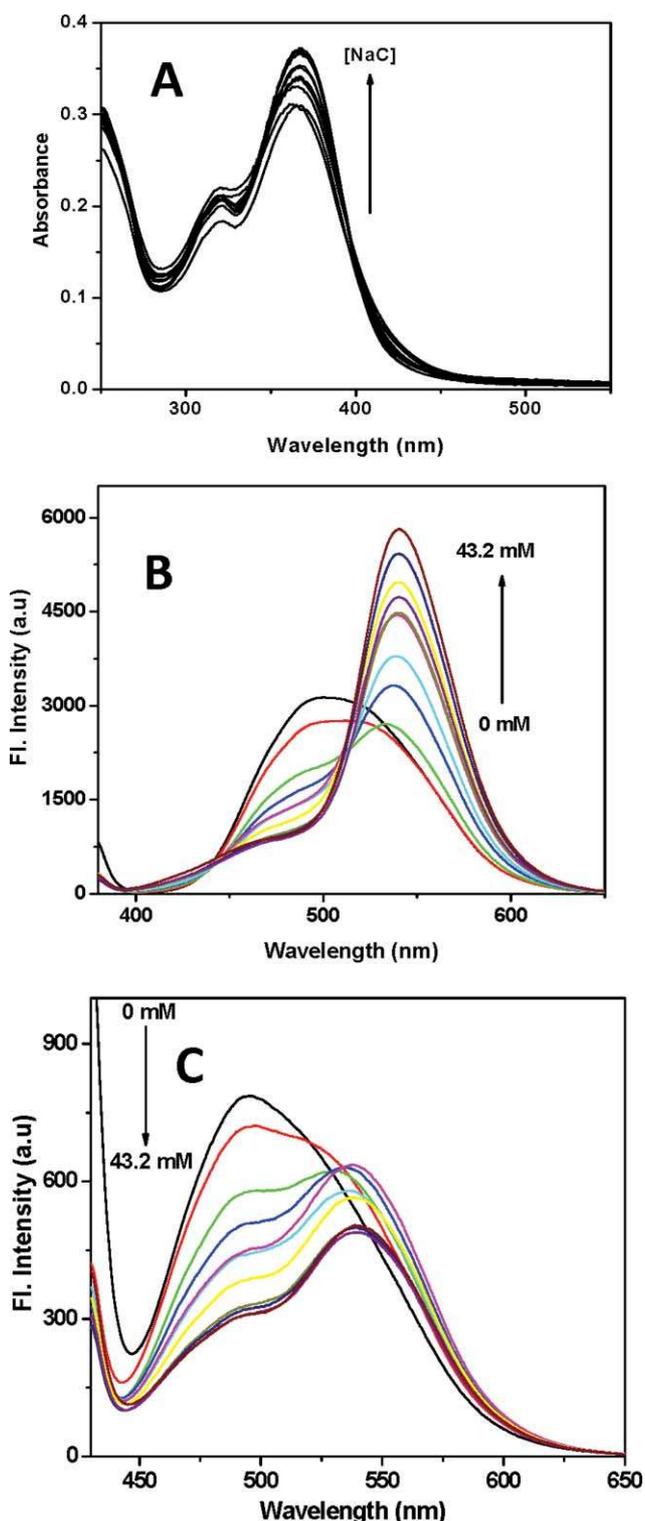
Fig. 1 (A) Absorption spectra of fisetin in varying concentration of NaC (0–43.2 mM). (B) Emission spectra of FN (λ_{ex} 369 nm). (C) Emission spectra of FA (λ_{ex} 418 nm). (T = 25 °C, pH = 7.4.)

increases with NaC concentration and correspondingly the absorbance at 418 nm decreases, with an isosbestic point observable at *ca.* 395 nm. Thus the enhanced absorption at 418 nm can be considered a weak band. These spectral features of fisetin in NaC

are comparable with those in homogeneous medium (Fig. S1, ESI†). The intense peak at 369 nm corresponds to FN (fisetin neutral), similar to those observed in non-polar solvents. The interesting observation of a weak band at 418 nm (Fig. 1A) is analogous to that observed in presence of alcoholic solvents (Fig. S1) and to the anion peak of the parent flavonoid, 3-hydroxyflavone (3-HF).[18,19] Hence the peak at 418 nm in the absorption spectra of fisetin can be attributed to its ground state anionic species ((FA)$_{GS}$).

The fluorescence spectra (Fig. 1B and Fig. 1C) of fisetin in NaC aggregates are measured at two excitation wavelengths; λ_{ex} 369 nm corresponding to the FN species (Fig. 1B) and λ_{ex} 418 nm corresponding to the (FA)$_{GS}$ species (Fig. 1C). The emission spectra of FN species (λ_{ex} 369 nm) show that it exhibits ESIPT, leading to the intense FT (fisetin tautomer) emission (λ_{em} 521–540 nm), comparable to the emission spectra of fisetin in non-polar solvents (Fig. S2) [5–11] and a less intense peak of (FA*)$_{ES}$ (excited state fisetin anion) at 490 nm along with the FN emission having a weak band at around 400 nm. FT emission increases gradually with increase in the NaC concentration, whereas the intensity of (FA*)$_{ES}$ decreases simultaneously and a minor increase with the FN emission is also noted. The emission spectra of (FA)$_{GS}$ with an excitation at 418 nm (Fig. 1C), show a prominent peak at 490 nm and presence of another peak overlaid along with the other peak. The second peak shifts from 521–542 nm with increasing concentration of NaC. The emission at 490 nm decreases along the NaC concentration and a small increase in the second peak is observed. The 490 nm peak is similar to that observed in alcoholic solvents (Fig. S2) and also comparable with that of the parent flavonoid, 3-HF.[18,19] So far, there has been just one report of emission peak at this region and it is assigned to the anion form of fisetin in the homogeneous medium.[6] Further evidence was obtained from a methanolic solution of fisetin in 0.1 M sodium methoxide, showing excitation peak at 414 nm and corresponding emission peak at 490 nm. Thus the peak observed at 490 nm (λ_{ex} 418 nm) in NaC is due to the emission of (FA)$_{GS}$. It is to be noted that the presence of the ground state anion in the anionic pesudo-micellar media of NaC can not be explained just by the pH of the medium; possibly the bile salt anions play the role of proton acceptor. The red shifted peak present in the emission spectra (Fig. 1C) is attributed to the FT species by comparing with the other spectra, Fig. 1B. The presence of FT emission with an excitation at 418 nm is an indication of the substantial presence of FN along with the (FA)$_{GS}$ in the ground state and thereby contributing to the characteristic FT emission. A normalized emission spectra (Fig. 1C) of (FA)$_{GS}$ indicates that as the NaC concentration increases, there is a decrease in the emission of (FA*)$_{GS}$ along with the increase in FT emission. The excitation spectra of FN and (FA)$_{GS}$ for their emission at 540 nm and 490 nm respectively are given in Fig. S3. Correlation of the absorption and emission measurements of fisetin in NaC aggregates to that of fisetin in homogeneous medium, suggests that the photophysical behavior of fisetin in NaC micellar media follows the processes involving ESIPT and ground state prototropic equilibria, provided in Scheme 1.

According to Scheme 1, there exists an equilibrium between FN and (FA)$_{GS}$ even in the ground state. The structure of fisetin anion has been assigned so as to show the first deprotonation at the hydroxyl group in the position *ortho* to C=O in the
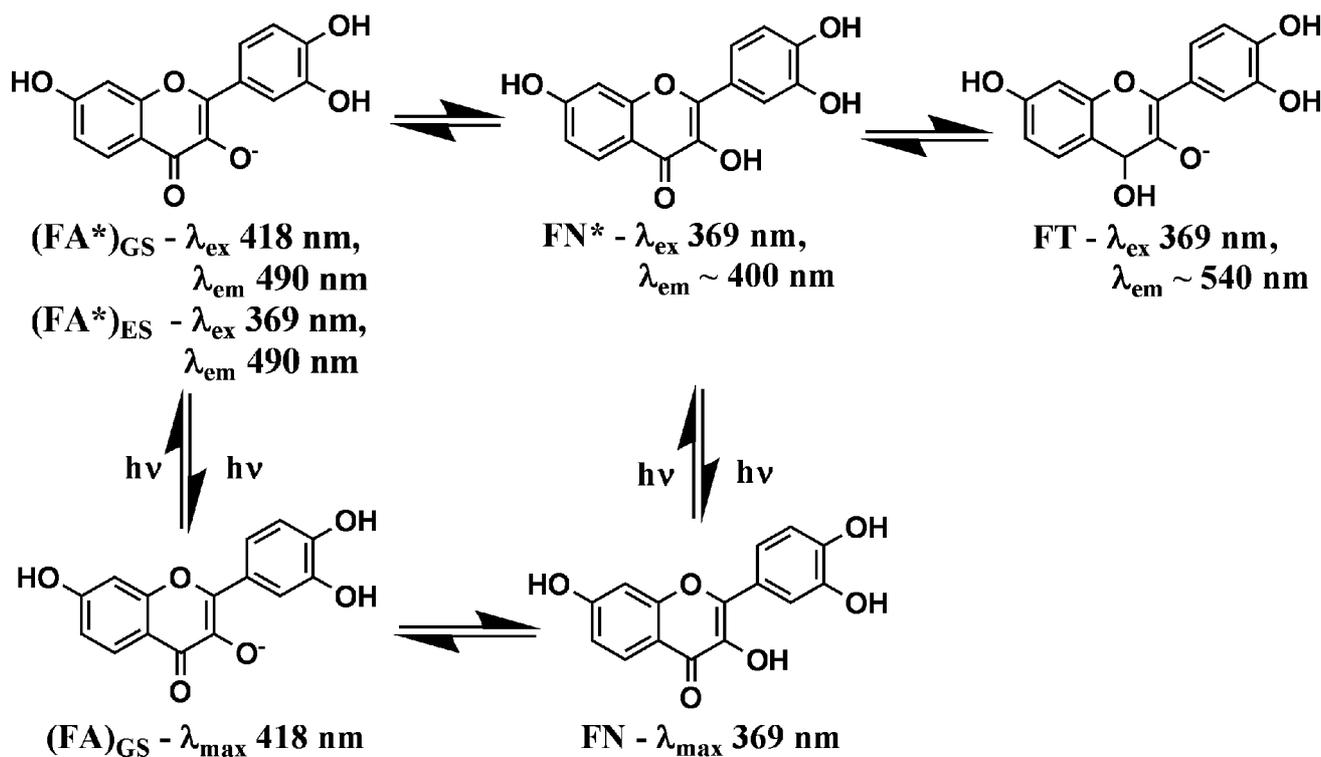
Scheme 1 Mechanism of ground state and excited state proton transfer reactions of fisetin in water (FN – fisetin neutral, FN* – excited state fisetin neutral, (FA)$_{GS}$ – ground state fisetin anion, (FA*)$_{GS}$ – excited species of ground state fisetin anion, (FA*)$_{ES}$ – excited state fisetin anion and FT – fisetin phototautomer).

fisetin molecule, by comparing it with the ground state anion structure of the parent 3-hydroxyflavone.[19] Moreover the excited state phototautomer does form by intramolecular proton transfer from this hydroxyl group.[6] Upon excitation at their characteristic wavelengths, FN species (λ_{ex} 369 nm) gives rise to FT, (FA*)$_{ES}$ and FN emission species, whereas the (FA)$_{GS}$ (λ_{ex} 418 nm) gives rise to (FA*)$_{GS}$ and FT emission. Scheme 1 also describes the prototropic equilibria of the various forms in the excited state, which leads to the highly overlaid emission spectra of FN and (FA)$_{GS}$ and hence there is no clear description of the emission peaks to each of the prototropic forms. However, the assignment of the emission peaks are rationalised by drawing analogy with the characteristic spectral features of the parent flavonoid, 3-HF.[18,19]

Various prototropic forms of fisetin in sodium cholate aggregates. The emission spectra of the two prototropic forms of fisetin (Fig. 1B and Fig. 1C) reveal two significant changes in the photophysical behavior of fisetin with the addition of NaC molecules. The first change is seen from the enhancement of the FT emission with the (FA*)$_{ES}$ emission decreasing correspondingly. The second is the nature of the spectral shifts of the FT and (FA*)$_{ES}$ emission peaks. Corroborating the absorption (Fig. 1A) spectral changes, the emission spectra shows that the (FA)$_{GS}$ is found to be present at the lower concentrations of NaC, *i.e.* below cmc range (12–16 mM) and as the NaC concentration increases, *i.e.* above cmc range (12–16 mM), the FN form becomes more prominent and subsequently the growth of FT emission is observed. This suggests that the (FA)$_{GS}$ form is the predominant prototropic form in primary NaC aggregates, and with increasing [NaC] and

formation of secondary aggregate the FN form of fisetin becomes the more favored. The progressive intensity enhancement and the wavelength shifts show gradual change as the NaC concentration increases and do not permit clear partitioning of the effects due to the primary and secondary aggregates. Since the secondary aggregates do not have any fixed aggregation number and their size keeps increasing as the concentration of bile salts, the increasing hydrophobicity of the media is due to the progressive increase in the size of the secondary aggregates.

The nature of shift associated with (FA*)$_{ES}$ and FT emission peaks, the emission spectra of FN (λ_{ex} 369 nm) in different NaC concentrations (Fig. 1B) were analyzed by deconvolution of these spectra (Fig. S4). The spectra contain a broad spectrum with large FWHM at the initial concentrations of NaC and as the NaC concentration increases, the subsequent spectra resolve into two distinct peaks. The emission spectra of fisetin in buffer medium (pH 7.4) were deconvoluted into two peaks corresponding to the well defined (FA*)$_{ES}$ emission peak at 478 nm in the blue-violet region and FT emission peak at 521 nm in the green region (Fig. S2). However, when fisetin is in NaC aggregates ([NaC] = 43.2 mM), the (FA*)$_{ES}$ peak shifts to 460 nm and the FT peak shifts to 544 nm. Collectively, the deconvoluted spectra show a blue shift associated with (FA*)$_{ES}$ emission peak and a red shift associated with FT along with the progressive increase in NaC concentration. These shifts in the emission maxima of (FA*)$_{ES}$ and FT in NaC aggregates can be correlated to those observed in the homogeneous medium (Fig. S2).

Owing to the charge on both the forms of fisetin molecules and also on NaC, the interaction between them is also expected

to be dependent on the charge and polarity surrounding the fluorophore. However, the persistence of $(FA)_{GS}$ form even in the highest concentration of NaC (43.2 mM) used here, suggests that the anion formation is not retarded by the negative charge on NaC aggregates. Thus the spectral features indicate that polarity of the medium affects the molecular association of fisetin with NaC aggregates. Correlating these absorption and emission measurements to that of fisetin in homogeneous medium suggests that the photophysical behavior of fisetin in NaC micellar media follows the processes involving ESIPT and ground state prototropic equilibria, provided in Scheme 1. It is to be noted that absorption and emission spectra of ground and excited state prototropic forms do overlap, and sometimes extensively. Thus exclusive excitation of a particular prototropic species is sometimes as difficult as getting the excitation spectrum of a particular prototropic species.

From these results, it appears that the fisetin molecule senses an increasing non-polar environment as the concentration of NaC increases. The shifts in the emission peak for both the forms of fisetin are due to the dipolar relaxation of the NaC molecules located at the interface. The interesting shifts in the emission maxima and the variations of emission intensity are conveniently represented by excitation emission matrix fluorescence (EEMF) (Fig. S5). The contour plots of fisetin in buffer medium (pH 7.4) is predominantly that of the $(FA)_{GS}$ form. As the concentration of NaC is increased, the contours shift gradually to the higher wavelength leading to a contour of FT fluorescence.

The fluorescence lifetime measurements at various emission wavelengths (λ_{em} = 480 to 560 nm) with a light source of 370 nm LED were carried out to evaluate the prototropism of fisetin in NaC aggregates (Table S1).

These fluorescence decays are fitted with a bi-exponential function and these decays depend on the emission wavelength. Following the assignment by Funes et al.,[6] the longer lifetime component can be attributed to $(FA^*)_{ES}$ form and the shorter to the FT species. The fluorescence lifetimes of both $(FA^*)_{ES}$ and FT species increase with the emission wavelengths. When an excited-state reaction or relaxation process is present and new species are created which emit at a different emission wavelength, the fluorescence decay dynamics will vary across the emission spectrum.[17] Thus the changes provided in Table S1, give another evidence for the prototropism of fisetin in NaC aggregates. These increasing lifetimes across the emission spectrum could also be interpreted in terms of dipolar relaxation of the micellar medium around the excited-state fluorophore.

Micellization of sodium cholate. From the emission spectral studies, it is clear that the ESIPT of fisetin is favored more as the concentration of NaC is increased, i.e. as the micellization of NaC proceeds. The variation of emission maxima and emission intensities of $(FA^*)_{ES}$ and FT are plotted against the varying concentration of NaC (Fig. 2) and therefore the fluorescence property of fisetin can be utilized to report the micellization of NaC molecules.

The plot in Fig. 2A shows a gradual blue shift in $(FA)_{GS}$ emission and a red shift in FT emission as NaC concentration increases. The results obtained here (Fig. 2A) along with earlier results (Fig. 1B) indicate that the fisetin molecule reports the change in NaC micellar medium to become non-polar as the NaC

concentration increases. The change in the relative population of $(FA^*)_{ES}$ and FT is further analyzed by correlating the emission intensity of $(FA^*)_{ES}$ (λ_{em} 490 nm; λ_{ex} 369 nm) and FT (λ_{em} 540 nm; λ_{ex} 369 nm) with increasing concentrations of NaC (Fig. 2B and C). The fluorescence intensity corresponding to a particular emitting species depends upon the population, the molar extinction coefficient (ε) and the fluorescence quantum yield (φ_f). In the present case, the light absorbing species at λ_{ex} 369 nm is the neutral form, thus the fluorescence intensity changes do not arise from ε. An indication of φ_f changes can come from the fluorescence lifetime (τ) values (Table S1) which are nearly constant for both $(FA^*)_{ES}$ and FT emissions. Thus bile salt induced population changes appear to be the primary reason for the fluorescence intensity changes. The observation of more intense FT emission at the expense of $(FA^*)_{ES}$ emission appears to be because of increased hydrophobicity of the bile salt aggregates that causes higher fraction of FN converting to FT, via FN* (Scheme 1, Fig. 2C). Even at the highest concentration of NaC used here ([NaC] = 43.2 mM), there is no complete conversion to the tautomer form. The cross-over point ([NaC] = 23 mM), given in Fig. 2C quantifies the concentration of NaC, where equal amounts of $(FA^*)_{ES}$ and FT are present within the NaC aggregates. The progressive hypsochromic shift of $(FA^*)_{ES}$ and bathochromic shift of FT cannot be explained if the environment of fisetin is purely aqueous. The only rational explanation can be provided in terms of increased hydrophobicity of the environment within the micellar aggregates. Thus, the plots provided here indicate that the multiple prototropism of fisetin closely follows the progressive micellization of NaC molecules.

At low concentrations of bile salts, they behave like an electrolyte and pH of the bile salt solution increases steadily as the bile salt micellization progresses. The role of bile salt aggregates in providing environment for the presence of multiple prototropic forms of fisetin, can be examined by taking different bile salts. The bile salts chosen for the present work are NaTC, NaDC and NaTDC. These three bile salts along with NaC are structurally different from one another. The structural variations of these bile salts are depicted in Fig. 1. In comparison to NaC, NaDC has one –OH group less on its steroidal moiety, NaTC has a side chain conjugation with taurine, an amino acid, and NaTDC has one –OH group less on its steroidal moiety along with a side chain conjugation. Therefore, the nature of the three bile salts with respect to their polarity differs from NaC as follows: NaDC in facial polarity, NaTC in terminal polarity and NaTDC in both facial and terminal polarity. Thus the deoxycholates (NaDC and NaTDC) are less polar than the cholates (NaC and NaTC). The alterations in their structure affect their micellization and solubilising properties. The micellization of bile salts are largely affected by their facial polarity and the presence of side-chain conjugation is very minimal. This is reflected in the cmc range of the bile salts; NaC = NaTC = 12–16 mM and NaDC = NaTDC = 4–6 mM.[16]

Photophysical behavior of different prototropic forms of fisetin in different bile salt media

The photophysical studies of fisetin in three bile salts (NaDC, NaTC and NaTDC) are carried out using the same procedure described earlier for fisetin in NaC aggregates. The absorption
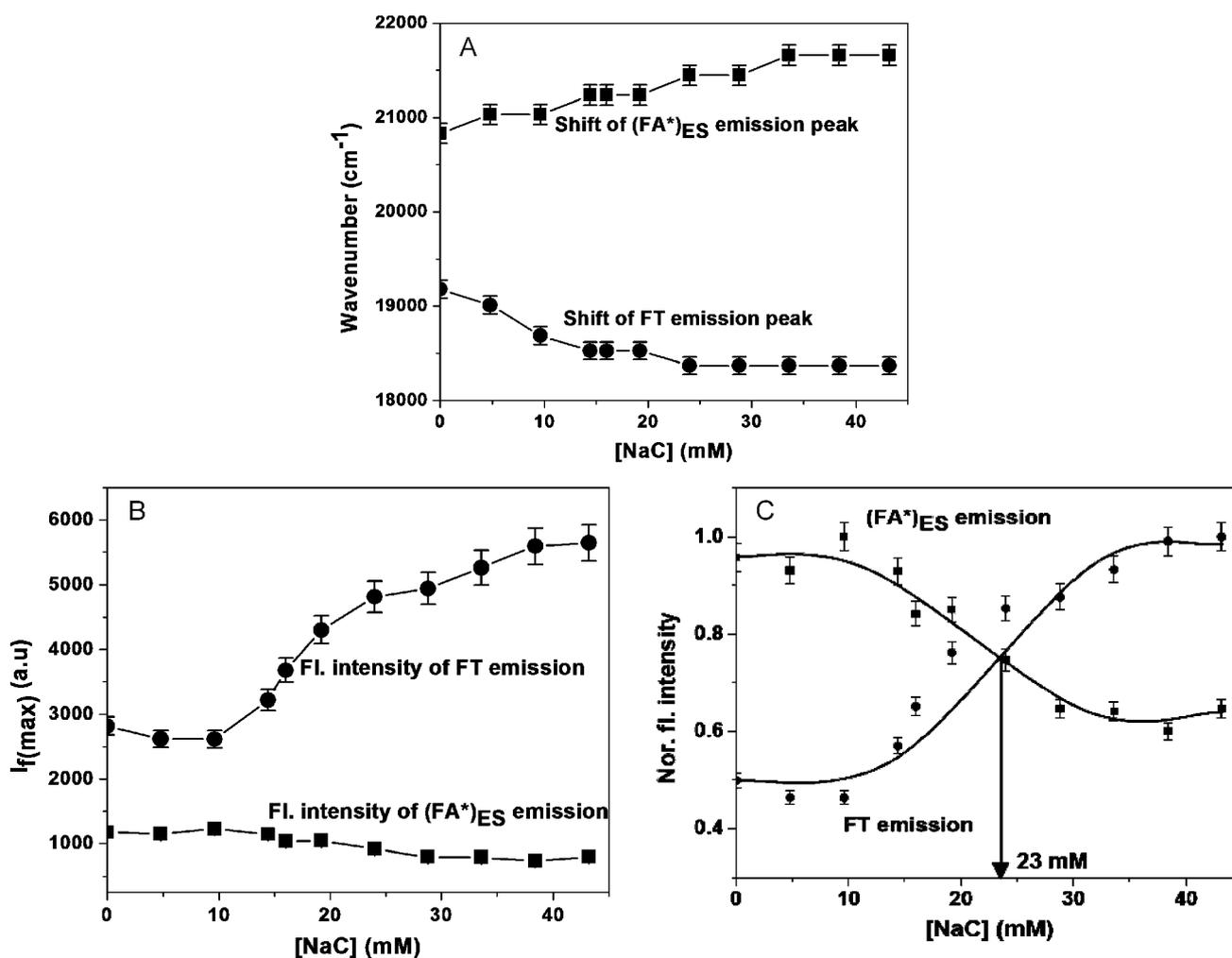
Fig. 2 (A) Dependence of $(FA^*)_{ES}$ and FT emission frequencies on NaC concentration. (B) and (C) Dependence of $(FA^*)_{ES}$ and FT emission intensities ([NaC] = 0 to 43.2 mM).

spectra (Fig. S6) and fluorescence spectra (Fig. S7) of fisetin in all these three bile salts are identical with those obtained in NaC molecules. The results obtained from these studies indicate that the presence of the $(FA)_{GS}$ species in the ground state, its spectral behavior and the unique shifts associated with the $(FA^*)_{ES}$ and FT emissions within the different bile salt media is true in case of all four bile salts including those observed in presence of NaC (Fig. 1). Small variations in presence of side-chain conjugated bile salts are observed and hence it can indicate minor effect due to these bile salts.

The intensity of $(FA^*)_{ES}$ emission increases in presence of the conjugated bile salts (NaTC and NaTDC). These results suggests that the FA species is more stabilized in presence of conjugated bile salts, since the $-SO_3^-$ group is less polar than the $-CO_2^-$ group, which is present at the side chain of bile salts. The normalized fluorescence intensity at emission peak maximum (λ_{ex} 369 nm) of both the species of fisetin in bile salts aggregates is correlated with the increasing concentrations of bile salts (Fig. 3). A cross-over point for each bile salt is obtained, *viz.* NaDC = 4.8 mM, NaTC = 29 mM and NaTDC = 9.8 mM. The higher cross-over concentration of the conjugated bile salts compared to that of the corresponding unconjugated bile salts clearly indicates that the

side-chain of these bile salts does play a role in their association with fisetin.
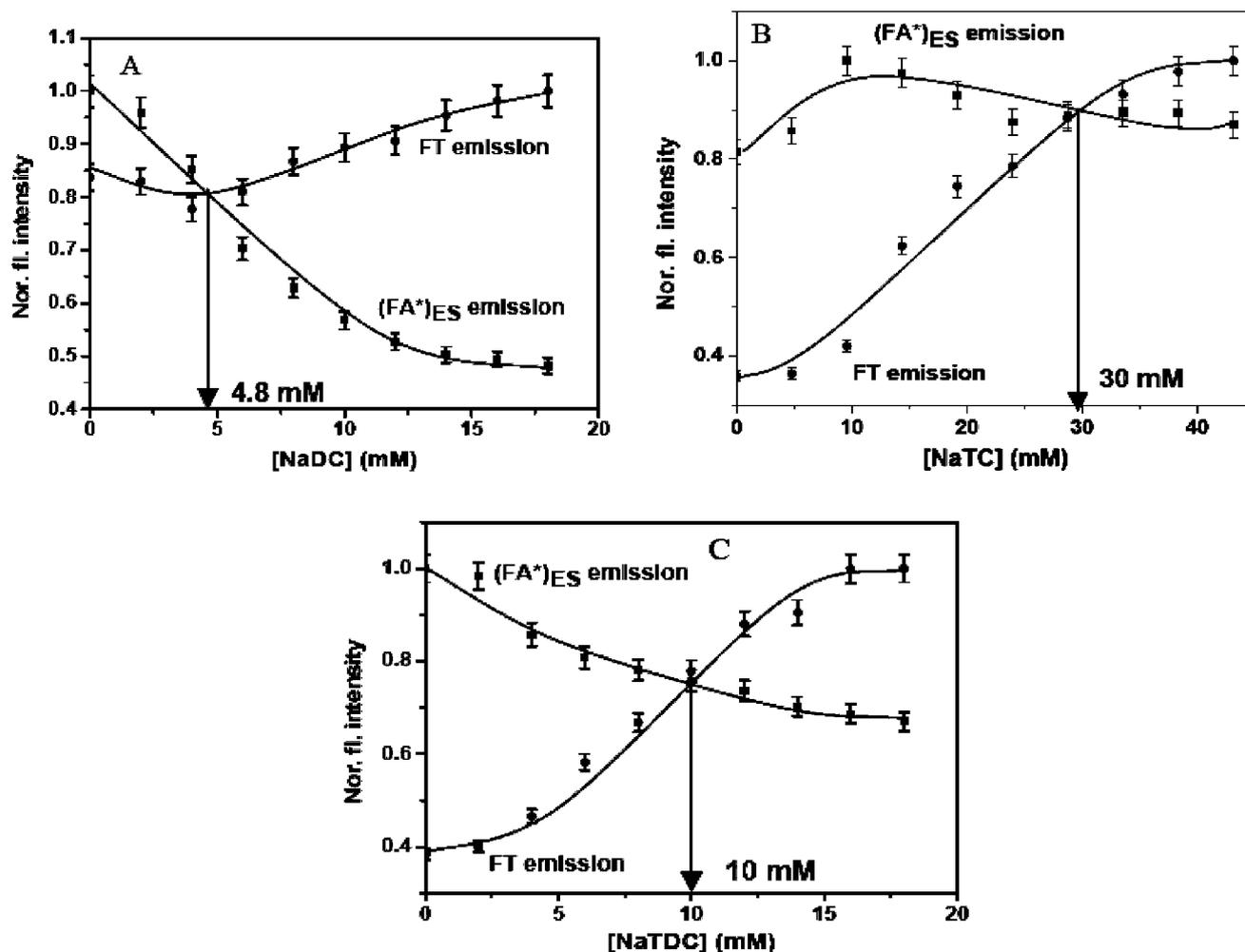
These studies give evidence for the hydrophobic steroidal face of the bile salts being involved in their interaction with both forms of fisetin, despite the possible electrostatic interaction between bile salts and both FA and FT species of fisetin. The results obtained from the absorption and emission spectral measurements of different prototropic forms of fisetin in various bile salt micelles, also support the hypothesis that the bile salt anion undergoes partial neutralization as their concentration is increased and results in increased hydrophobicity. The association of fisetin with all four bile salts are further studied with the steady state fluorescence anisotropy and fluorescence lifetime measurements.

Steady state fluorescence anisotropy and lifetime measurements

The steady state fluorescence anisotropy (r_{ss}) values for $(FA)_{GS}$ (λ_{ex} 418 nm, λ_{em} 490 nm) and FT (λ_{ex} 369 nm, λ_{em} 540 nm) forms of fisetin in buffer medium (pH 7.4) and different bile salt aggregates ([NaDC] = [NaTDC] = 18.0 mM and [NaC] = [NaTC] = 43.2 mM) are given in Table 1. Both $(FA^*)_{GS}$ and FT emissions have lower

Table 1 Steady state fluorescence anisotropy (r_{ss}) of two prototropic forms of fisetin in buffer medium (pH 7.4) and NaC aggregates ([NaC] = 43.2 mM). The errors are within ± 0.01

System	Phototautomer (FT) (λ_{ex} 369 nm, λ_{em} 540 nm)	Anion (FA)$_{GS}$ (λ_{ex} 418 nm, λ_{em} 490 nm)
Fisetin-buffer medium (pH 7.4)	0.04	0.04
Fisetin-NaC	0.14	0.16
Fisetin-NaDC	0.16	0.16
Fisetin-NaTC	0.12	0.16
Fisetin-NaTDC	0.17	0.15

Fig. 3 Dependence of (FA*)$_{ES}$ and FT normalized fluorescence intensity at emission peak maximum on bile salt concentration at λ_{ex} 369 nm, showing the crossover point for each bile salt. (A) NaDC (0–18 mM), (B) NaTC (0–43.2 mM) and (C) NaTDC (0–18 mM).

r_{ss} values at pH 7.4 and gradually increase as the concentration of bile salt increases (Table 1).

This suggests that both forms of fisetin are conveniently located within the rigid micellar environments of bile salts, where the rotational motion of the fluorophore is restricted. The equilibrium between (FA)$_{GS}$ and FN exists within a same environment of bile salt aggregates, which can be interpreted from the near similar values of r_{ss} for both the forms of fisetin. The fluorescence lifetime measurements of fisetin in different bile salts are obtained by using an excitation source of 370 nm LED for both (FA*)$_{ES}$ emission (λ_{em} 490 nm) and FT emission (λ_{em} 540 nm). The fluorescence decays are fitted with a bi-exponential function and measured for different concentrations of all four bile salts. The fluorescence lifetimes

along with their individual components are given in Table 2 to show the change in the fraction of (FA)$_{GS}$ and FT forms of fisetin.

The emission decay profile of both species measured in their respective emission peak shows enhancement of the FT emission and also persistence of (FA)$_{GS}$ emission. The increase in τ_1 of (FA)$_{GS}$ emission is seen only in conjugated bile salts, whereas τ_2 increases in all four bile salts. Both τ_1 and τ_2 of FT emission increases with the increasing concentrations of all four bile salts and the magnitude of the increase in lifetimes is more in conjugated bile salt aggregates. Even though the change in all the lifetimes are minor, the change in the individual contribution of each lifetime shows that there is a certain equilibrium between the anionic

Table 2 Fluorescence lifetime values of $(FA^*)_{ES}$ and FT emission of fisetin in buffer (pH 7.4) and different bile salt concentrations. $\lambda_{ex} = 370$ nm

Micellar system	[Bile salt]/mM	Anion (λ_{em} 490 nm)		Phototautomer (λ_{em} 540 nm)	
		$\tau_1 (\alpha_1)$	$\tau_2 (\alpha_2)$	$\tau_1 (\alpha_1)$	$\tau_2 (\alpha_2)$
Fisetin-buffer (pH 7.4)	—	0.7 (0.83)	2.6 (0.16)	0.9 (0.94)	3.3 (0.05)
Fisetin-NaC	4.8	0.7 (0.81)	2.7 (0.19)	1.0 (0.87)	3.2 (0.12)
	16.0	0.7 (0.79)	3.0 (0.20)	1.0 (0.51)	3.2 (0.48)
	43.2	0.7 (0.73)	3.3 (0.26)	1.0 (0.45)	3.3 (0.54)
Fisetin-NaDC	2.0	0.7 (0.83)	2.7 (0.17)	0.9 (0.93)	2.9 (0.07)
	6.0	0.7 (0.82)	2.7 (0.18)	0.9 (0.62)	3.1 (0.38)
	18.0	0.7 (0.80)	2.7 (0.20)	1.0 (0.56)	3.2 (0.44)
Fisetin-NaTC	4.8	0.8 (0.79)	2.7 (0.21)	1.1 (0.82)	3.2 (0.18)
	16.0	0.8 (0.75)	3.2 (0.24)	1.5 (0.58)	3.3 (0.42)
	43.2	0.8 (0.69)	3.5 (0.30)	1.5 (0.47)	3.9 (0.53)
Fisetin-NaTDC	2.0	0.9 (0.81)	2.8 (0.19)	1.0 (0.53)	3.0 (0.47)
	6.0	0.9 (0.73)	3.0 (0.26)	1.2 (0.51)	3.9 (0.49)
	18.0	0.9 (0.64)	3.2 (0.36)	1.4 (0.41)	4.0 (0.59)
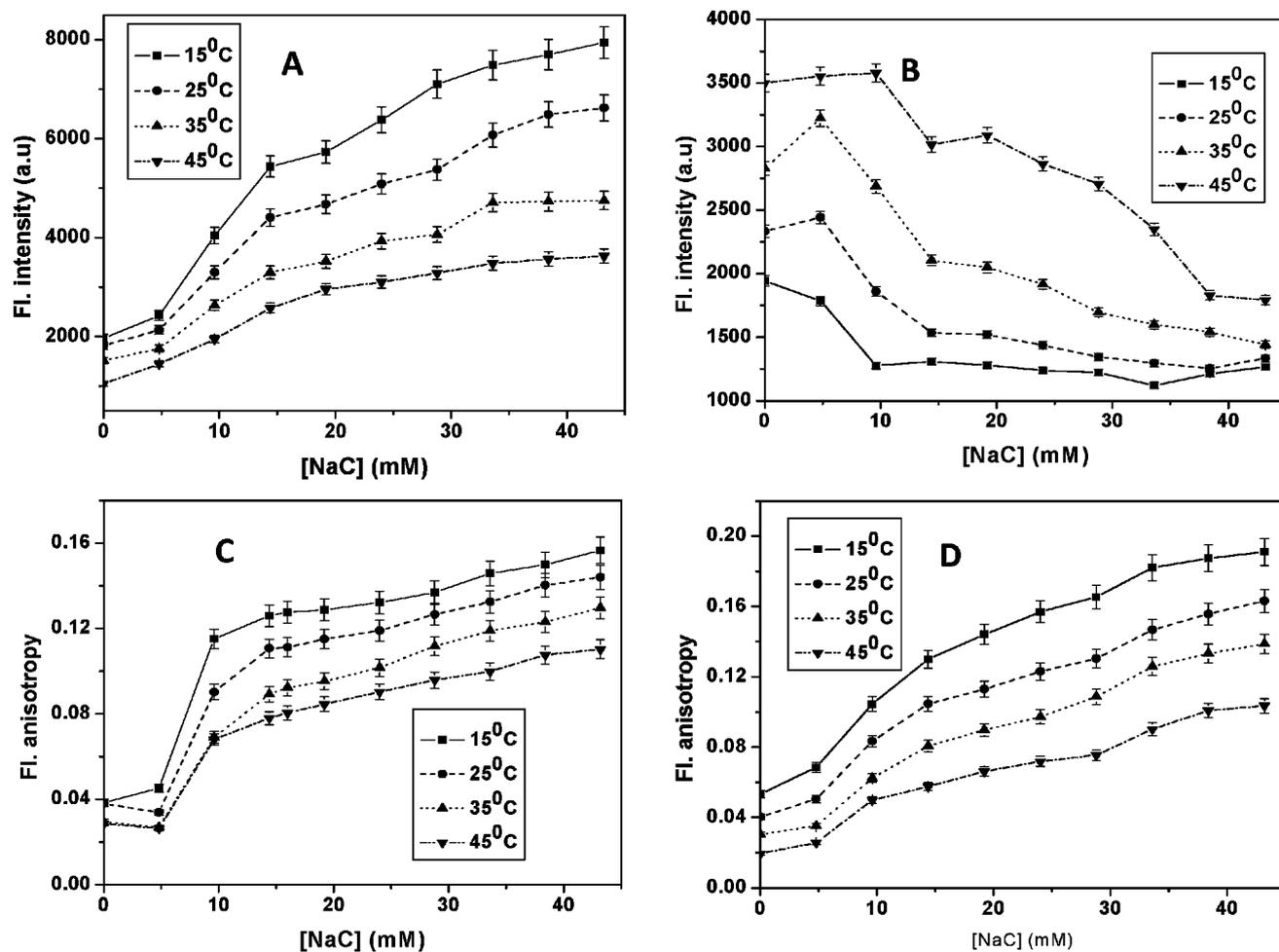
Fig. 4 Dependence of (A) FT emission intensity (λ_{ex} 369 nm and λ_{em} 540 nm) and (B) $(FA)_{GS}$ emission intensity (λ_{ex} 418 nm, λ_{em} 490 nm), (C) FT fluorescence anisotropy (λ_{ex} 369 nm and λ_{em} 540 nm) and (D) $(FA)_{GS}$ fluorescence anisotropy (λ_{ex} 418 nm, λ_{em} 490 nm) on NaC concentrations at different temperatures.

and tautomer fisetin species within the bile salt aggregates. The microheterogeneity around the fluorophore is expected to be rather distributed. Hence, fluorescence lifetime data analysis involving a few component fit may be approximate in describing the physical situation. Thus the lifetime data reported can only be considered as supporting data.

Effect of temperature

The effect of temperature on the interaction between fisetin and bile salts are carried out within the temperature range 15–45 °C.[20,21] The photophysical parameters such as fluorescence emission intensities (Fig. 4A, Fig. 4B and Fig. S8) and steady state

fluorescence anisotropy (Fig. 4C, Fig. 4D and Fig. S9) of (FA)$_{GS}$ (λ_{ex} 418 nm, λ_{em} 490 nm) and FT (λ_{ex} 418 nm, λ_{em} 490 nm) are utilized to probe the molecular association of fisetin along with the four bile salts at different temperatures.

With increasing temperature, the fluorescence intensity of the FT emission decreases in presence of all four bile salts, even in a narrow temperature range of 15–45 °C. Such a strong dependence of prototropism is usually not expected in homogeneous media. Hence the observed decrease in the FT emission could be due to temperature induced bile salt structural changes, like micellar disaggregation, which can lead to an increase in the polarity of the bile salt media. The fluorescence anisotropy values of both the species in all the four bile salts decreases with decreasing concentration of bile salts. Thus these results strongly suggest that both the forms of fisetin experiences restrictive rotation within the micellar core of bile salt media. The almost similar values of the both fisetin species are observed here and this indicates that they are located within a similar micro-heterogeneous environment.

The retardation of FT and enhancement of (FA)$_{GS}$ emission intensities follow the progressive micellization behavior of bile salts, through an initial formation of dimeric primary aggregates and then larger secondary aggregates of bile salts, in all the temperatures used here. Temperature dependence of the progressive micellization of bile salts suggests that the dimeric aggregation is unaffected, while the larger aggregates are further grown with increasing temperature.[21] As the temperature increases, there is decrease in secondary aggregate formation, i.e. lowering of micellization due to the hydrogen bond distortions. Thus at higher temperatures, the polarity of bile salt media increases, leading to an unfavorable condition for the FT formation and hence assists the formation of (FA)$_{GS}$ by participating in intermolecular hydrogen bonding with the fisetin molecules. This observation is conveniently visualized in the EEMF spectra of fisetin in bile salt aggregates at higher temperature of 45 °C (Fig. S10). At the highest concentration of NaC, the contour of FT emission is blurred at this temperature, as compared to its characteristic contour observed at 25° C (Fig. S5) and the proportion of FA is seen to be more. The results suggest that the fisetin molecule senses the disaggregation of bile salts aggregates as temperature increases. The results obtained from the fluorescence intensity and fluorescence anisotropy of two forms of fisetin at different temperatures provides corroborating evidence for the hydrophobic nature of interaction between fisetin and bile salts.

Conclusions

This study reports the observation of ground state fisetin anion ((FA)$_{GS}$) in alcohols and bile salts for the first time. The hypsochromic shift of (FA*)$_{ES}$ emission and the bathochromic shift of FT emission provides a convenient way of monitoring the progressive increase of medium nonpolarity when the bile salt concentration is increased. The spectral features of these prototropic forms give a valuable evidence for the neutralization of the bile salt anions as their concentration is increased. The absorption and fluorescence spectra of fisetin in varying concentration of NaC show that not only multiple prototropic forms, namely FN, (FA)$_{GS}$ and FT are present within bile salt aggregates, but also

that their relative concentrations change with change of bile salt concentration.

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