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Partitioning of Prototropic Species of an Anticancer Drug Ellipticine in Bile Salt Aggregates of Different Head Groups and Hydrophobic Skeleton: A Photophysical Study to Probe Bile Salt as Multisite Drug Carrier

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Abstract: The entrapment of neutral and cationic species of an anticancer drug namely ellipticine and their dynamic features in different bile salt aggregates have been investigated for the first time using steady state and time-resolved fluorescence spectroscopy. Because ellipticine exists in various prototropic forms in physiological condition, we performed a comparative photophysical and dynamical studies of these prototropic species in different bile salts varying their head groups and hydrophobic skeletons. We found that the initial interaction between ellipticine and bile salt is governed by the electrostatic forces where cationic ellipticine is anchored to the head groups of bile salts. Bile salts of conjugated head groups are better candidates to bind with the cationic species than the non-conjugated once. The fact implies that binding of cationic species to different bile salts depends on the pKa of corresponding bile acids. The hydrophobic interaction dominates at higher concentration of bile salts due to formation of aggregates and results in entrapment of neutral ellipticine molecules according to their hydrophobicity indices. Thus bile salt acts as a multisite drug carrier. The rotational relaxation parameters of cationic ellipticine were found to be head group dependent and number of hydroxyl groups in the hydrophilic surface of bile salts. Cationic ellipticine exhibits a faster rotational relaxation in the tri-hydroxy bile salt aggregates than in di-hydroxy bile salts. We interpreted this observation by the fact that tri-hydroxy bile salt hold more number of water molecules in their hydrophilic surface offering a less viscous environment for ellipticine compared to di-hydroxy bile salts. Surprisingly, the neutral ellipticines display almost same rotational relaxation in all the bile salts. The observation indicates that after intercalation inside the hydrophobic pocket neutral ellipticine molecules experience similar confinement in all the bile salts.

Introduction: Ellipticine is a pyridocarbazole type plant alkaloid which exhibits cytotoxic activity against tumor cells. Methoxyellipticine lactate and 2-methyl-9-hydroxylellipticiniumacetate exhibit a significant biological activity.[1-6] The biological action of these pyridocarbazoles results in direct binding to DNA. They induce protein associated DNA strand break by trapping Topoisomerase II. The cytotoxicity of ellipticine is supposed to be primarily related to two modes of action: (i) intercalation into DNA and (ii) inhibition of DNA topoisomerase II activity.[3-8] The size and planar molecular structure of ellipticine resembles those of a purine-pyrimidine complementary base pair which provides favourable conditions for intercalation in DNA. Recently, ellipticine and structurally related compounds were reported to have application in the treatment of obesity and tested for human pre-AIDS treatment in association with other drugs.[9-10] A substantial research work has been devoted to understand the biological activity, sequence selectivity, and metabolism of ellipticine for several years.[11-21] Recently, the photophysical properties of ellipticine has been explored by several groups. Fung and co-workers reported photophysical properties of ellipticine in different solvents varying the polarity and hydrogen bonding.[22] The authors found a large Stokes' shift (10000 cm^{-1}) in polar solvents compared to that (8900 cm^{-1}) in non polar solvents. Miskolczy et al. reported that in methanol ellipticine takes up a proton from the solvent in its first excited state.[23-24] Very recently Banerjee and co-workers reported that in methanol the second emission band occurs due to excited state reaction.[25] The study suggests that excited state reaction involves solvent reorganization around ellipticine to form a "cyclic" solvated species which facilitates a rapid proton transfer and the two emission bands arise from the normal and tautomeric forms. This phenomenon was supported by excitation spectra of ellipticine in various solvents. The authors obtained a rise component of around 2 ns in methanol which explicitly proves that ellipticine undergoes a tautomerization in its excited state. Our group previously reported the entrapment and subsequent interconversion of different prototropic species in aqueous reverse micelles and non-aqueous microemulsion.[26]

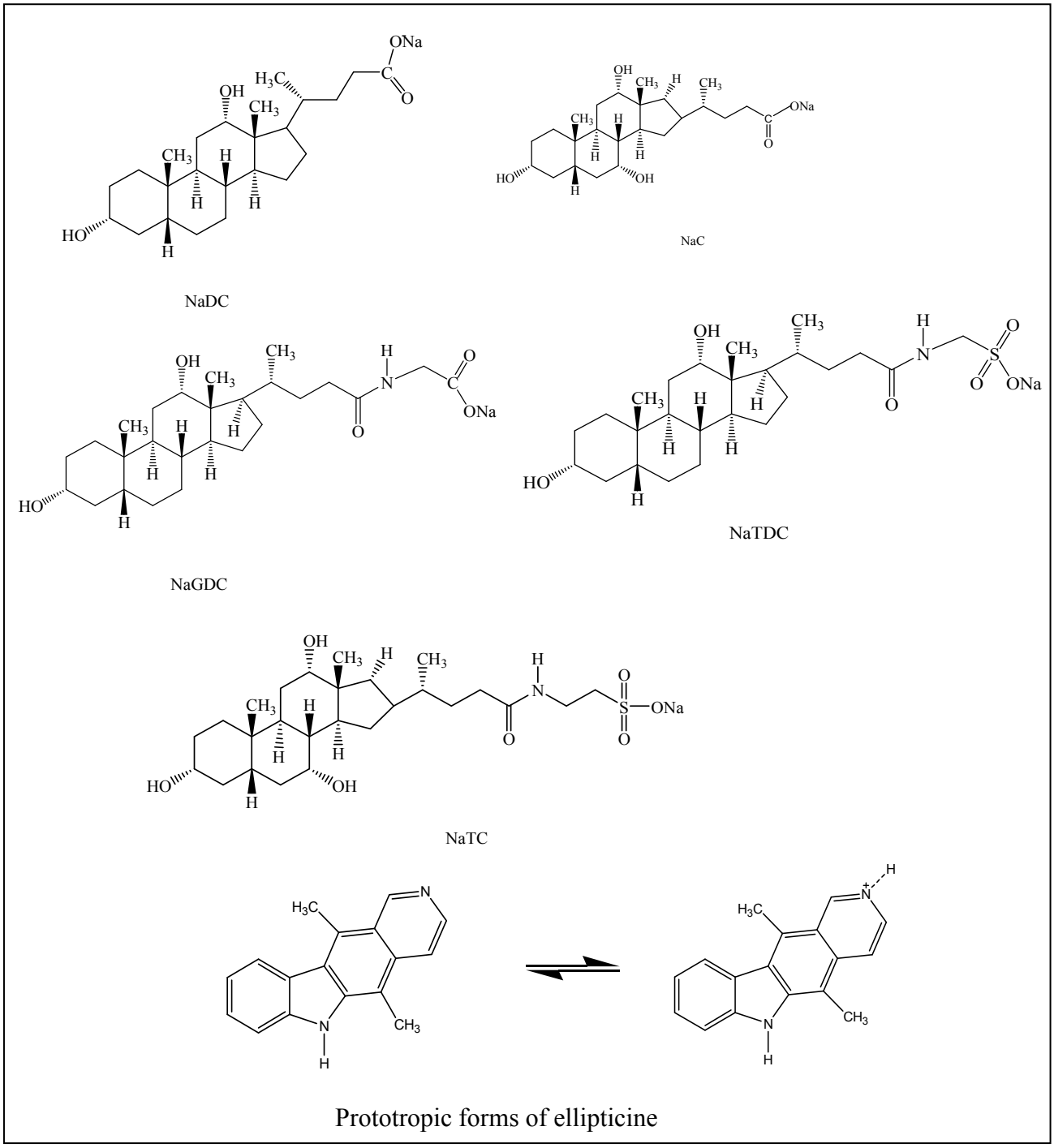
Ellipticine exists as protonated or deprotonated species in aqueous medium depending on the pH.[26-33] At pH value below 7.4, due to protonation of pyridine like nitrogen cationic species are prevalent.[26] Studies in living cells have revealed that ellipticine exists both in neutral and protonated forms in aqueous cytoplasm, but only in its protonated form in the nucleus.[32] The major shortcomings in usage of neutral ellipticine as pharmaceuticals are its toxicity and low

solubility (~0.6×10^{-7} M) in water but cationic ellipticine is relatively more soluble in water than neutral ellipticine. To surmount the trouble of low solubility of ellipticine in aqueous media, the drug was attached to polymers, peptides, micelles and liposomes.[34-39] Therefore, such amphiphillic systems are desired which can modulate the different prototropic species of ellipticine. In this case, bile salt aggregates may be potential supramolecular host systems that can carry both hydrophobic and hydrophilic guest molecules of suitable size and shape because of the presence of both types of binding sites under varying experimental conditions, such as concentration, pH, and ionic strength of the surrounding medium.[40-44] 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.[40-48] This structure leads to a unique aggregation pattern, accounting for their solubilization of both hydrophobic and hydrophilic solutes. Bohne and co-workers extensively carried out the study of host guest complexation of different probe molecules in bile salt aggregates.[49-53] Consequently bile salts have received much attention as drug delivery media. These bioactive molecules are synthesized in the liver from cholesterol and play an important role in the solubilization of lipids in the intestine, which allows them to be used as a potential drug delivery system.[54-62] Very recently, Miranda and co-workers demonstrated the existence of the two types of aggregates (primary/secondary) at different concentrations using fluorescent dansyl derivative of sodium cholate It was shown that bile salt aggregates are suitable for carrying both hydrophobic and hydrophilic drug molecules due to the presence of these two different types of binding sites.[59-61] Miranda and co-workers showed that cholic acid aggregates can be used as effective drug carrier drug carriers. They have shown that hydrophobic drugs like naproxen and its methyl ester derivative can be entrapped in the hydrophobic pocket of bile salts.[62] The self-assemblies of bile salts are of particular interest from the biological point of view because of their unique ability to solubilize various biologically active organic guests including many sparingly water-soluble drug molecules.[47-62] Moreover, the formation of inclusion complexes in such aggregates helps to control the selectivity of various chemical reactions such as photoinduced reactions, enzymatic reactions and complexation reactions. Due to presence of different tunable binding sites, bile salt aggregates are found to be interesting host systems capable of carrying both hydrophobic and hydrophilic guest molecules depending on the structure and size of the guests. Recently, Sarkar and co-workers has carried out extensive host guest interaction study in different bile slats.[55, 63-64] This group reported

photophysics of different molecules like curcumin, $BP(POH)_2$ and HAN in different bile salt systems. Collective and self-diffusion coefficients of sodium taurodeoxycholate and taurocholate in D_2O system have been reported as a function of ionic strength and bile salt concentration by quasielastic light scattering.[65] Bhattacharya and co-workers reported the dynamics of probe molecules in bile salt aggregates.[66-67]

In the present paper, we would like to report photophysical and photodynamical properties of different prototropic species of ellipticine entrapped in the hydrophobic and hydrophilic sites of bile salts of different head groups and hydrophobic skeletons. The present study involves five bile salts namely sodium salt of deoxycholte (NaDC), cholate (NaC), glycodeoxycholate (NaGDC), taurodeoxycholate (NaTDC) and taurocholate (NaTC). The structural formulae of bile salts are shown in scheme 1. It is revealed from scheme 1 that the head groups of NaDC and NaC are akin but NaC contain an extra hydroxyl group in its hydrophilic surface. The hydroxyl groups make NaC less hydrophobic as compared to NaDC. Again NaDC, NaGDC and NaTDC have same number of hydroxyl groups in the hydrophilic surface but their head groups are different. While NaDC possesses an unconjugated head group (CH_2-COO^-), NaGDC and NaTDC have conjugated head groups -CO-NH-CH_2-CO_2^- and -CO-NH-CH_2-SO_3^- respectively. Owing to conjugation, head groups of NaTDC and NaGDC are supposed to be better proton donor than the head group of NaDC (i.e. the bile acids with conjugated head groups are stronger acid than bile acids with unconjugated head groups.). On the other hand, head group of NaTC (-CO-NH-CH_2-SO_3^-) is same as that of NaTDC. However, extra hydroxyl group in their hydrophilic surface offers an additional hydrophilicity in NaTC as compared to that in NaTDC.

Because ellipticine exists in protonated and deprotonated species in aqueous solution, therefore; by varying the head group and hydrophilic skeleton of bile salts, we would be able to tune the photophysics and dynamics of the ellipticine molecules as per our aspiration. In the present study we explored the entrapment of neutral and cationic species in hydrophobic and hydrophilic skeleton respectively and observed the effect of head groups as well as hydrophobic skeleton on the binding, partition and confinement of ellipticine molecules.



Scheme 1. *Structural formulae of different bile salts and prototropic form of ellipticine*

Experiment: Bile salts were purchased from sigma-aldrich and were used as received. Bile salt solutions were prepared by dissolving in 0.2 M NaCl solution as described by Sarkar et al.[55] The stock solutions of bile salt, especially for concentrated solution were heated at 50^0 C to avoid gel formation. Required amount of stock methanolic solution of ellipticine was taken in a volumetric flask so that final concentration of ellipticine becomes ~ 10^{-7} M for a specific volume of bile salt. The solution was kept few hours for the encapsulation of ellitpicine into the microenvironment of bile salt.

Steady state absorption spectra were taken in a Varian UV-Vis spectrometer (Model: Cary 100). Emission spectra were taken in a Fluoromax-4p fluorimeter from Horiba Yovin (Model: FM-100). The samples were excited at 375 nm. All the measurements were done at 25^0 C.

For the time resolved studies, we used a picosecond time correlated single photon counting (TCSPC) system from Horiba Yovin (Model: Fluorocube-01-NL). The experimental setup for TCSPC has been described elsewhere. The samples were excited at 375 nm using a picosecond diode laser (Model: Pico Brite-375L). The signals were collected at magic angle (54.70^0) polarization using a photomultiplier tube (TBX-07C) as detector. The instrument response function of our setup is ~140 ps.

The amplitude weighted average lifetime was calculated using following equation

$$\langle \tau \rangle = \sum_{i=1}^{n} a_i \tau_i \qquad (1)$$

Where τ_i are the fluorescence lifetimes of various fluorescent components and a_i are the normalized pre-exponential factors.

Result and discussion:

We already stated that ellipticine exists in two prototropic species.[26] Cationic species dominate at low pH due to protonation of the nitrogen atom on pyridine moiety and neutral species are prevalent at higher pH. Both the species simultaneously exist in physiological condition. The detailed emission properties of ellipticine were reported in aqueous medium at different pH in our earlier publication.[26] Ellipticine shows two bands around 440 nm and 540 nm at physiological condition (pH~ 7.40). We assigned the bands at 440 and 540 nm wavelengths to neutral and cationic species respectively. Therefore, any change in the intensity at 540 nm is attributed to cationic-anionic interaction which is electrostatic in nature. On the other hand,

change in the intensity at 440 nm is due to the hydrophobic interaction. The fluorescence quantum yield of cationic species of ellipticine was reported to be around 0.002 while the neutral species has a quantum yield around 0.0004.[26,35] Addition of bile salts to aqueous solution of ellipticine enhances the intensity at 540 nm as well as at 440 nm (Figure 1) along with a significant increment in the overall solubility of ellipticine.

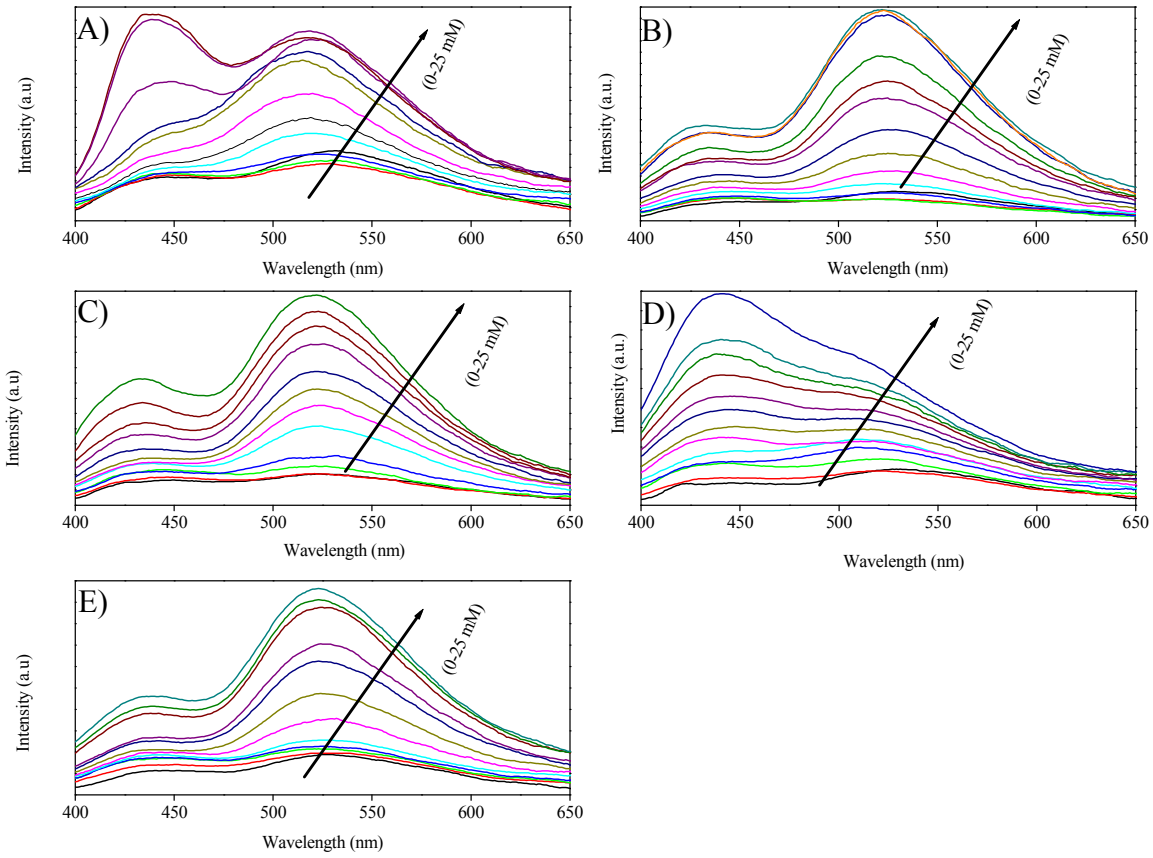


Figure 1. *The emission spectra of ellipticine in presence of increasing concentration of different bile salts (0-25 mM) (A) NaDC (B) NaTC (C) NaTDC (D) NaC (E) NaGDC.*

The rise in intensity at 540 nm indicates that the binding of cationic species with bile salts. On the other hand increase in intensity at 440 nm indicates that the neutral probe molecules are entrapped in the hydrophobic pocket of bile salt. The emission band of neutral species gradually shifts to the blue end. The extent of blue shift is greater in NaDC compared to other bile salts. This observation is consistent with earlier report that NaDC is more hydrophobic than other blue shifts. The ratios of intensity of neutral species to that of cationic species (i.e. I_{440}/I_{540}) are 1.12,

1.81, 0.60, 0.48 and 0.42 for NaDC, NaC, NaTDC, NaGDC and NaTC respectively. In our earlier publication,[26] we have shown the excitation spectra of neutral and cationic ellipticine in aqueous solution at pH~10 and pH~2. In the present case, we provided normalized excitation spectra at 440 and 540 nm in presence of 15 mM NaDC (Figure 2A). By comparing the excitation spectra in presence of bile salt with excitation spectra of neutral and cationic ellipticine as reported earlier,[26] it is confirmed that the increment in the intensity in emission spectra at 440 nm is due to entrapment of the neutral species while the increment at 540 nm is due to entrapment of cationic species in bile salt aggregates.

To gain an insight regarding the binding of neural and cationic species with bile salts, we deconvoluted the emission spectrum into cationic species and neutral species by a combination of lognormal functions of the following form (Figure 2B)

$$I(\nu) = I \exp[-\ln 2(\frac{\ln[1 + 2b(\nu - \nu_p)/\Delta]}{b_1})^2] \qquad (2)$$

where ν_p, I, Δ, and b stand for peak frequency, peak height, width parameter and asymmetric parameters respectively. All the fluorescence spectra were scaled by a λ^2 factor before the lognormal fitting of the respective spectra to obtain the frequencies. The area under each of the curve corresponding to neutral and cationic species was used to estimate the quantum yield using the following equation

$$\phi_S = \phi_R \left(\frac{n_S^2}{n_R^2} \right) \left(\frac{I_S}{I_R} \right) \left(\frac{1 - 10^{-0.5 A_R}}{1 - 10^{-0.5 A_S}} \right) \qquad (3)$$

We used Quinine sulphate dihydrate in 0·05 M H_2SO_4 as reference (ϕ_R = 0·508). In equation 3, n_s and n_R represent refractive index of the sample (S) and reference solution (R) respectively, I is the integrated emission intensity, and A is the absorbance. The plot of $\phi_{cationic}/\phi_{neutral}$ as a function of concentration of different bile salts (Figure 2C) reveals that $\phi_{cationic}/\phi_{neutral}$ initially decreases till 2-2.5 mM and then increases, reaches a maximum and then decreases for all the bile salts except NaTC. The initial decrement in $\phi_{cationic}/\phi_{neutral}$ may be attributed to the fact that very low concentration of bile salt increases the pH of the solution and this converts cationic species into the neutral species. This factor may be responsible for decrement in $\phi_{cationic}/\phi_{neutral}$ ratio in all bile salts. The rise in $\phi_{cationic}/\phi_{neutral}$ signifies that a strong interaction between

ellipticine and bile salt is dominated by electrostatic forces where the cationic ellipticine species are anchored to the negatively charged head groups of bile salts. The electrostatic interaction dominates till a certain concentration of bile salts. The hydrophobic interaction dominates at higher concentration of bile salt due to formation of aggregates which results in entrapment of neutral ellipticine in the hydrophobic cavity of aggregates. It is notable that aggregates of bile salts of non-conjugated head groups (for e.g. NaDC and NaC) display the drop in $\phi_{cationic}/\phi_{neutral}$ (i.e. dominance of hydrophobic force) at lower concentration than in bile salts having conjugated head groups. This observation indicates that unconjugated bile salts provide a more hydrophobic environment compared to conjugated bile salts. Moreover, it is observed that ellipticine exhibits higher $\phi_{cationic}/\phi_{neutral}$ value in conjugated bile salt aggregates (like NaTC and NaTDC) than that in non-conjugated aggregates. This fact indicates that the cationic species of ellipticine is more stabilized in conjugated bile salt aggregates. It is also possible that the neutral species turn into the cationic species in the head group region of conjugated bile salts. In that case the cationic species seems to be stabilized in the head group region and neutral species are stabilized in the hydrophobic surface.

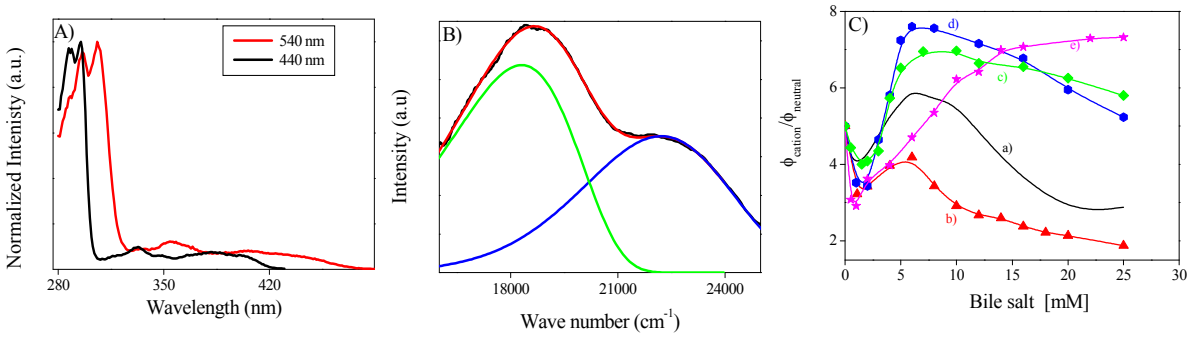


Figure 2. *A) Excitation spectra of ellipticine in 15 mM NaDC at 440 nm and 540 nm. (B) Deconvolution of emission spectrum of ellipticine by a combination of lognormal functions. (C) The plot of* $\phi_{cationic}/\phi_{neutral}$ *as a function of different concentration (0-25 mM) of different bile salts (a) NaDC (b) NaC (c) NaGDC (d) NaTDC (e) NaTC.*

We interpreted the above findings in the light of structural difference of bile salts along with different parameters like hydrophobicity indices[68-70] and dissociation constant (pK_a) values of

corresponding bile acids.[71-72] We listed the hydrophobicity indices of different bile salts and the pka of the corresponding acids in Table 1.

Table 1. *Hydrophobicity indices of different bile salts and pK_a of corresponding bile acids*

System	No. of -OH groups in hydrophilic site	Hydrophobicity indices	Head groups	pK_a of corresponding bile acids
NaDC	2	0.72	$-CH_2-CH_2-CO_2^-$	6.20
NaGDC	2	0.65	$-CH_2-CO-NH-CO_2^-$	4.80
NaTDC	2	0.59	$-CH_2-CO-NH-SO_3^-$	<2
NaC	3	0.13	$-CH_2-CH_2-CO_2^-$	5.20
NaTC	3	0.00	$-CH_2-CO-NH-SO_3^-$	<2

It is seen from Table 1 that the pK_a of conjugated bile acids is less than non-conjugated one. Therefore, head group of conjugated bile salt would be more dissociative than non-conjugated ones hence will produce more number of anions. We shall start our discussion with photophysics of ellipticine in three different bile salts namely NaDC, NaTDC and NaGDC. These three bile salts have same number of hydroxyl groups in their hydrophobic skeleton but differ in their head groups. NaDC possesses an unconjugated head group ($-CH_2-CH_2-COOH$) while NaTDC and NaGDC possess conjugated head groups $-CO-NH-CH_2-COOH$ and $-CO-NH-CH_2-SO_3H$ respectively. It is revealed from Table 1 that conjugated head groups like $-CO-NH-CH_2-COOH$ and $-CO-NH-CH_2-SO_3H$ are stronger acid than unconjugated ones like $-CH_2-CH_2-COOH$. Thus it implies that head group of NaDC is less acidic compared to its analogous bile salts NaTDC and NaGDC although these three bile salts have same number of hydroxyl group in their hydrophobic moiety. The hydrophobicity indices in Table 1 reveal that NaDC is more hydrophobic as compared to NaTDC and NaGDC. Therefore NaTDC and NaGDC exhibit higher affinity towards cationic species of ellipticine yielding higher $\phi_{cationic}/\phi_{neutral}$ as compared to NaDC.

Now we would like to compare entrapment of cationic and neutral species in NaGDC and NaTDC. Although NaGDC is more hydrophilic than NaTDC, Table 1 reveals that pKa of taurine group is less (< 2) than that of glycine (4.80). Therefore, due to taurine head group NaTDC is better candidate to capture the cationic ellipticine and results in larger increment of

$\phi_{cationic}/\phi_{neutral}$ values. Figure 2 reveals that $\phi_{cationic}/\phi_{neutral}$ values increases sharply in NaTC and does not decrease till 25 mM. This result is contradictory to that of NaTDC in which $\phi_{cationic}/\phi_{neutral}$ drops after a certain concentration. Although, these two bile salts have same head group, but NaTC possess an additional hydroxyl group in its hydrophilic skeleton and that makes NaTC more hydrophilic than NaTDC. The observation suggests that hydrophobic interaction between ellipticine and NaTC aggregates is much weaker compared to that between ellipticine and NaTDC. However, the above argument does not hold when we compare the results in NaDC and NaC aggregates. It is observed that ellipticine exhibits a much smaller $\phi_{cationic}/\phi_{neutral}$ value in NaC as compared to that in NaDC aggregates although NaC is more hydrophillic than NaDC. We are not sure about the origin of this anomalous result. This anomalous result perhaps comes from the fact that NaC is less rigid compared to NaDC, thus allow more number of neutral ellipticine molecules to penetrate inside NaC compared to that in NaDC. Moreover, the critical micellear concentration of NaC is higher (16 mM) compared to that of NaDC (4 mM) which results in entrapment of more number of neutral ellipticine. This could be the probable reason of observed anomalous result in NaC.

In the present perspective, the partition coefficient of the neutral species can be a measure of the hydrophobicity of different bile salts. We, therefore, estimated the partition coefficient of neutral ellipticine using the following formulae (Figure 3).[73]

$$\frac{1}{F} = \frac{55.6}{(K_P F_0 L)} + \frac{1}{F_0} \qquad (4)$$

where F_0 and F are fluorescence intensities of ellipticine molecules in aqueous and in bile salts phase, respectively, L is the bile salt concentration and the molar concentration of water was considered to be 55.6 M.

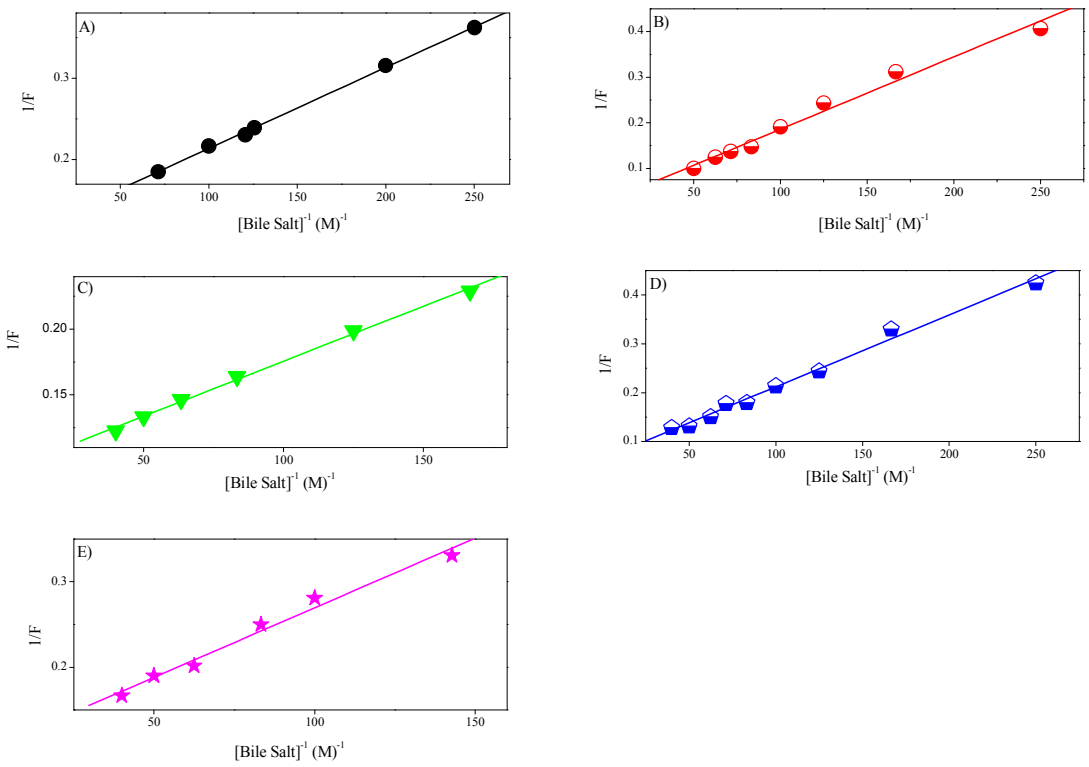


Figure 3. *Double reciprocal plot of the intensity of ellipticine with respect to concentration of different bile salts.*

The partition coefficients of the neutral species in different bile salts are reported in Table 2.

Table 2: *Partition coefficient and free energy change of neutral species of ellipticine in different bile salt aggregates*

Bile salt	Partition coefficient, K_P (10^3)	Binding Enegry, -ΔG (kJ mol^{-1})
NaDC	7.50	22.10
NaTC	0.98	17.05
NaTDC	5.90	21.50
NaC	2.50	19.30
NaGDC	3.60	20.30

It is revealed that the partition coefficient between water and bile salt aggregates is highest in NaDC while the partition coefficient is lowest in NaTC. The order of the partition coefficient is in order of the hydrophobicity of bile salts. Therefore, it is conclusive that neutral species of ellipticine is entrapped in the bile salt aggregates according to the hydrophobicity while the cationic species are attached to the head groups of bile salts according to number of hydroxyl group and acidity of the head group region. Figure 4 provides a cartoon representation indicating the solubilization of neutral ellipticine in the hydrophobic pocket of micellar aggregates of bile salts and entrapment of cationic species in the hydrophilic surface.

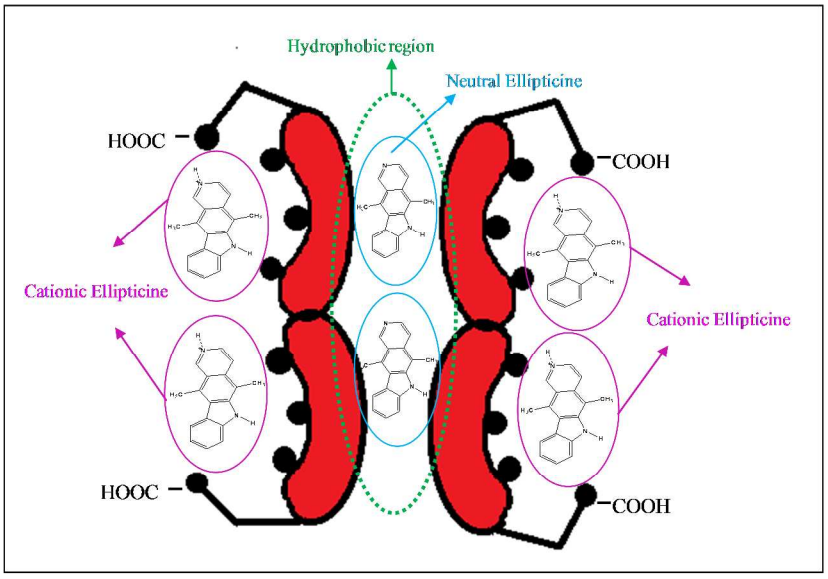


Figure 4. *Entrapment of neutral and cationic species of ellipticine in hydrophobic and hydrophilic site of bile salt aggregates.*

Time resolved studies: We already mentioned that ellipticine exists in cationic and neutral species in aqueous solution. Therefore, we took decays at 540 and 440 nm to measure the lifetime of cationic species and neutral species respectively. We reported the lifetime of both species in our earlier publications.[26-35] The lifetime of ellipticine at 540 nm consists of components around 1.80 (88%) and 5.94 ns (12%). The component around 1.80 ns was attributed to the cationic species of ellipticine which is predominant at 540 nm emission band. The other component perhaps comes from the zwitterionic species in the solution. Table 3 reveals that, the shorter component i.e. τ_1 remains unchanged with addition of bile salts to

aqueous solution of ellipticine while a longer component (τ_2) is generated which increases with increasing concentration of bile salts. The increment in longer component is ascribed to the ellipticine cation binding to head groups of bile salt and eventually formation of bile salt aggregates. We plotted τ_1 and τ_2 as a function of concentration of different bile salts (Figure 5). It is observed that the maximum increment in longer component takes place in NaTC while the least increment takes place in NaDC and NaC. The longer components were found to be 7.50, 12.95, 12.0, 7.50 and 8.9 ns in case of NaDC, NaTC, NaTDC, NaC and NaGDC respectively. Notably, τ_2 values are almost same in NaDC and NaC, although they are widely different in their hydrophobicity indices. Again a similar increment in τ_2 components is observed in NaTDC and NaTC aggregates despite of wide difference in the hydrophobicity indices of NaTDC and NaTC. Therefore, we conclude that the order of increment in the longer time component is not governed by the hydrophobicty indices of bile salts. It depends on the nature of the head groups of bile salt. Therefore, we have taken pK_a of bile salts into account for the interpretation of lifetime component. Table 1 reveals that NaDC and NaC possess same head groups which render similar pK_a for these two bile acids. Therefore, both NaDC and NaC offer similar environment in their head group region towards ellipticine and results in similar changes in τ_2. On the other hand NaTC and NaTDC have same head group. Therefore, ellipticine cation experiences similar binding in the head group region of these bile salts. Thus we observe a similar increment in τ_2 in these two bile salts. This assumption is further validated if we look at the amplitudes of τ_2 which, in the present case are found to be directly related to the pK_a values of bile acids. Bile acids with lower pK_a will be more dissociative and hence produce more number of anions which can bind with the cationic ellipticine species. Table 1 reveals that maximum change in population of the longer component takes place in NaTC (40%) while minimum takes place in case of NaDC (23%) and NaC (21%). The population of τ_2 depends directly on the pKa of corresponding bile acids. It implies that higher the dissociation constant of bile acids higher would be the number of cations bound to head groups region. To illustrate this fact, we plotted a_2/a_1 as a function of concentration of different bile salts (Figure 5c).

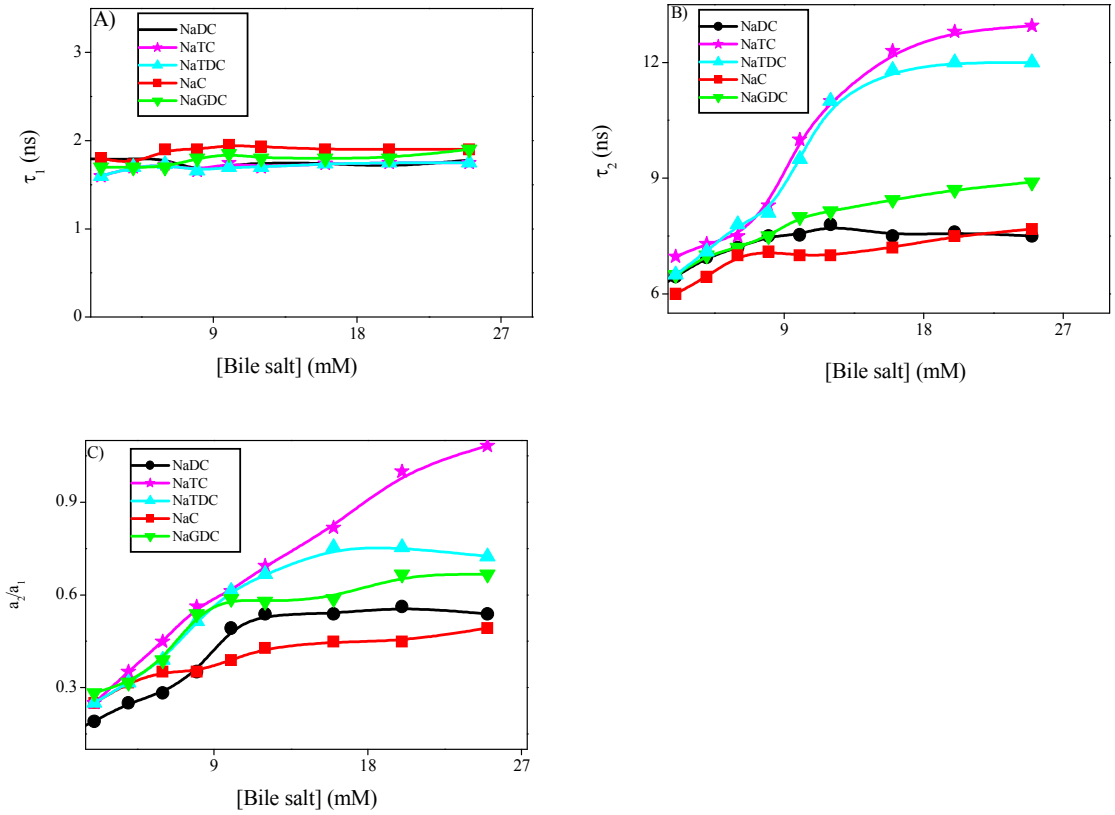


Figure 5. *(A) Shorter (τ_1), (B) longer (τ_2) lifetime components and (C) ratio of population (a_2/a_1) of lifetime components of ellipticine in presence of different bile salt concentration.*

The increment in a_2/a_1 values follows the same order of τ_2. In the earlier section, we already mentioned that binding of cationic species depends on the hydrophilicty of bile salts as well as the protonation ability of corresponding bile acids. Therefore, the lifetime components and amplitudes of the cationic species unambiguously establish this fact that entrapment of cationic species of ellipticine takes place in bile salt according to the pKa of corresponding bile acids.

Table 3. *The decay components of ellipticine in presence of different bile salts at 520 nm.*

System	a_1 (%)	a_2 (%)	τ_1 (ns)	τ_2 (ns)	$\langle\tau\rangle$ (ns)	χ^2
Conc. of NaDC						
0	0.88	0.12	1.80	5.94	2.30	1.20
2	0.84	0.16	1.79	6.45	2.53	1.21
4	0.80	0.20	1.79	6.95	2.82	1.20
6	0.78	0.22	1.8	7.20	2.99	1.15
8	0.74	0.26	1.650	7.50	3.17	1.16
10	0.67	0.33	1.720	7.53	3.63	1.23
12	0.65	0.35	1.75	7.80	3.87	1.10
16	0.65	0.35	1.75	7.50	3.76	1.05
20	0.64	0.36	1.70	7.60	3.82	1.20
25	0.65	0.35	1.78	7.50	3.78	1.10
Conc. of NaTC						
2	0.80	0.20	1.60	6.97	2.67	1.16
4	0.74	0.26	1.70	7.30	3.16	1.23
6	0.69	0.31	1.73	7.50	3.52	1.10
8	0.64	0.36	1.66	8.30	4.00	1.05
10	0.62	0.38	1.75	10.00	4.88	1.20
12	0.59	0.41	1.70	11.00	5.51	1.10
16	0.55	0.45	1.74	12.30	6.50	1.16
20	0.50	0.50	1.75	12.80	7.27	1.23
25	0.48	0.52	1.75	12.95	7.57	1.10
Conc. of NaTDC						
2	0.80	0.20	1.60	6.50	2.58	1.20
4	0.76	0.24	1.70	7.10	3.00	1.15
6	0.72	0.28	1.73	7.80	3.43	1.16
8	0.66	0.34	1.66	8.10	3.85	1.23
10	0.62	0.38	1.70	9.50	4.67	1.10
12	0.60	0.40	1.70	11.00	5.42	1.20
16	0.57	0.43	1.74	11.80	6.00	1.15
20	0.57	0.43	1.75	12.00	6.15	1.20
25	0.58	0.42	1.75	12.00	6.00	
Conc. of NaC						
2	0.80	0.20	1.8	6.00	2.64	1.20
4	0.76	0.24	1.73	6.44	2.86	1.10
6	0.74	0.26	1.90	7.00	3.23	1.16
8	0.74	0.26	1.90	7.10	3.25	1.23
10	0.72	0.28	2.00	7.00	3.40	1.10
12	0.70	0.30	1.932	7.00	3.45	1.10
16	0.69	0.31	1.90	7.20	3.54	1.19
20	0.69	0.31	1.9	7.50	3.64	1.25
25	0.67	0.33	1.9	7.68	3.81	1.30

Conc. of NaGDC						
2	0.78	0.22	1.70	6.50	2.75	1.23
4	0.76	0.24	1.70	7.0	2.97	1.48
6	0.72	0.28	1.70	7.20	3.24	1.45
8	0.65	0.35	1.80	7.50	3.79	1.19
10	0.63	0.37	1.85	8.0	4.12	1.25
12	0.65	0.35	1.75	8.15	3.99	1.20
16	0.63	0.37	1.56	8.44	4.10	1.23
20	0.60	0.40	1.68	8.70	4.49	1.10
25	0.60	0.40	1.9	8.90	4.70	1.10

Unlike lifetime components at 540 nm, life time components at 440 nm do not follow any particular trend of hydrophobicity of the bile salts. We summarized the lifetime components in Table S1 in the supplementary material. In aqueous solution the life time of ellipticine at 440 nm consists of picosecond component around 0.400 ns (90%) and a longer component around 3.40 ns (10%). Addition of bile salts causes an enhancement in the picosecond component almost by two times along with an increase in the nanosecond component. The decrease in the population of the picosecond component and subsequent increment in population of longer component indicate that neutral species are trapped in the hydrophobic site. However, we observe that the change in population follows the trend of the hydrophobicity of the bile salts.

We estimated the rotational relaxation of ellipticine in different bile salts (Figure 6). The time resolved anisotropy was described with the following equation:

$$r(t) = r_0\left[\beta_{fast}\exp\left(-t/\phi_{fast}\right) + \beta_{slow}\exp\left(-t/\phi_{slow}\right)\right] \quad (5)$$

where $r(t)$ is the rotational relaxation correlation function. r_0 is the limiting anisotropy and ϕ_{fast} and ϕ_{slow} are the individual rotational relaxation time and β_{fast} and β_{slow} are the amplitude of rotational relaxation time.

In aqueous solution ellipticine exhibits a single exponential decay with rotational relaxation time around 180 ps. In bile salt aggregates, the rotational relaxation is found to be bi-exponential consisting of a picosecond component and a nanosecond components. The picosecond component originates from the ellipticine molecules in aqueous phase while nanosecond component is attributed to the ellipticine molecules held in the bile salt aggregates. The rotational relaxation parameters are summarized in Table 4. We have estimated the

microviscosity of the hydrophilic region with the following equation considering the fact that ϕ_{slow} arises from the ellipticine molecules bound to the head group region

$$\phi = \frac{\eta_m V}{kT} \quad (6)$$

Here, η_m is the microviscosity and V is the volume of the rotating molecular system. Hence, any change in ϕ could come from a change in either of the two factors (η, V). We have already reported the volume of ellipticine in our earlier publication. Table 4 reveals that the microviscosity is reasonably higher for conjugated head groups with less number of hydroxyl groups. This observation again corroborates well with the assumption that the bile salts having three hydroxyl groups can hold more number of water molecules which results in lower viscosity as compared to that in bile salts having two hydroxyl groups.

Table 5. *Analytical parameters of rotational relaxation time of ellipticine in different bile salt aggregates*

Conc. bile salts	r_0	β_{fast} (%)	β_{slow} (%)	ϕ_{fast} (ns)	ϕ_{slow} (ns)	χ^2	$\langle\eta_m\rangle$ (cP)
0	0.30	1.00		0.180		1.0	
25 mM NaDC	0.38	0.66	0.33	0.160	1.54	1.1	30.00
25 mM NaTC	0.38	0.60	0.40	0.160	1.71	1.15	33.50
25 mM NaTDC	0.40	0.34	0.66	0.184	2.71	1.1	52.50
25 mM NaGDC	0.40	0.52	0.48	0.160	2.01	1.0	39.00
25 mM NaC	0.40	0.50	0.50	0.187	1.20	1.2	23.50

We would like to start our discussion by comparing rotational relaxation in the bile salts which possess same head groups but differ in number of hydroxyl groups in their hydrophilic surface. It is revealed from Table 5 that ϕ_{slow} is higher in NaDC as compared to that in NaC. This may be attributed to the fact that NaDC possesses two hydroxyl groups in its steroidal moiety while NaC possesses three hydroxyl groups. Therefore, NaC can hold more number of water molecules. Thus the hydrophilic region of NaC is less vicous which reduce the frictional force as compared to that in NaDC. This perhaps results in faster rotational relaxation of ellipticine in NaC as compared to that in NaDC. A similar conclusion can be drawn if we compare ϕ_{slow} in NaTC and NaTDC. Ellipticine experiences less rigidity in NaTC due to an additional hydroxyl group as compared to that in NaTDC and hence exhibits faster rotational relaxation time.

Now we need to address the rotational relaxation in bile salts which have same number of hydroxyl group in the hydrophilic surface but differ in head groups. There are three bile salts namely NaDC, NaTDC and NaGDC which are same in number of hydroxyl groups in the hydrophilic site but differ in head groups. We found that ellipticine exhibits faster rotational relaxation in NaDC compared to that in NaGDC and NaTDC. NaGDC and NaTDC possess unsaturated head groups which have lower pKa hence higher dissociation constant than that of saturated head groups of NaDC. Thus NaTDC and NaGDC are capable of holding more number of ellipticine cations leading to higher population of longer component. This fact may be responsible for the higher rotational relaxation time in NaGDC and NaTDC. Interestingly, the longer component is higher in NaTDC than that in NaGDC. The taurine group is more acidic than glycine group. Therefore, NaTDC is better candidate to capture cationic species of ellipticine. Thus higher partitioning of cationic ellipticine takes place in NaTDC.

One inference can be drawn from the above discussion that partitioning of cationic species of drugs between aqueous phase and miceallar surface not only depends on the nature of the head groups but also on the number of hydroxyl group on the hydrophilic surface. Interestingly, we did not find any residual anisotropy which indicates absence of any restricted rotation in bile salt aggregates. In this context the comparison of global dynamics (i.e. rotation of the bile salt) and local dynamics (i.e. rotation of the probe molecules inside the bile salt aggregates) should be addressed. Maitra et al. reported that hydrodynamic radius of NaC is around 1.2 nm.[74] The global rotational relaxation time was estimated to be around 1.2 ns obtained from the following equation

$$\phi_m = \frac{4\pi R_h^3 \eta_m}{3kT} \qquad (7)$$

This value is close in the range of the longer component (1.20 ns to 2.71 ns) of ellipticine in bile salt aggregates. The observation implies that global rotational motion of bile salt aggregates has a significant contribution to the overall rotation of probe. Therefore, the rotational dynamics of ellipticine is governed by the two factors (i) microvscosity felt by the ellipticine and (ii) the golbal rotation of micellar aggregates.

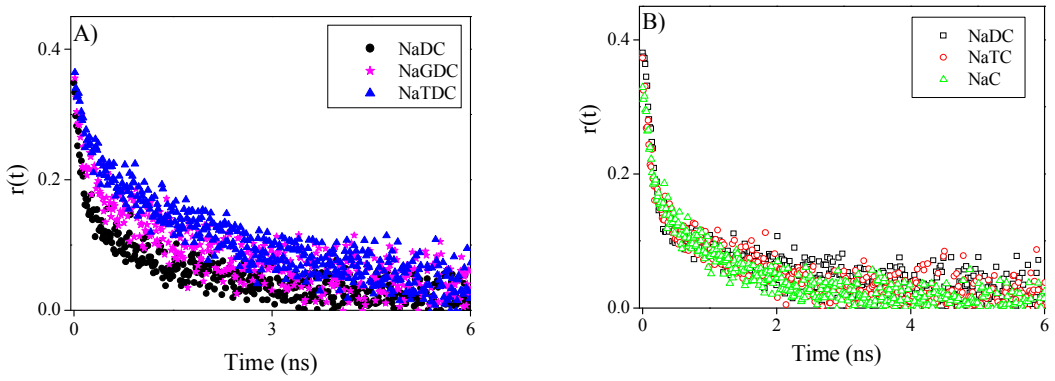


Figure 6. *(A) Fluorescence anisotropy decays of cationic ellipticine in different bile salts at 540 nm.(B) Fluorescence anisotropy decays of neutral ellipticine in different bile salts aggregates at 440 nm.*

Unlike cationic ellipticine, when we measured rotational relaxation time at 440 nm, neutral ellipticine molecules exhibit almost same rotational relaxation time in different bile salt aggregates (Figure 6B). The fact implies that after intercalation, ellipticine experiences a similar environment inside the hydrophobic pocket of bile salt. We have already shown in Figure 4 that neutral ellipticine preferably form complex to the hydrophobic region bile salt. One interesting point that Moitra et al. reported is DPH displays a single exponential correlation function which comes from the hydrophobic pocket of bile salts.[74] It was reported that DPH being very hydrophobic mostly are entrapped in the hydrophobic pocket. In the present case, Figure 6 reveals a fast component (data are not shown) which is likely to be originated from neutral ellipticine in the aqueous phase. The observation implies partitioning of neutral ellipticine in aqueous and hydrophobic phase. The full intercalation in the hydrophobic surface is not possible due formation of hydrogen bonding with water as well as bile salt head groups.

Conclusion: In summary, we have shown the nanoconfinement of different prototropic species of anticancer drug ellipticine in several bile salt aggregates through hydrophobic and hydrophilic interaction. The neutral species of the drug molecules were found to be partitioned according to the hydrophobicity indices of the bile salt. On the other hand, cationic species are attached to the head group region of the bile salt aggregates through electrostatic interaction. Our study revealed that initially bile acts like electrolytes and electrostatic interaction dominates over the

hydrophobic interaction. The extent of binding of bile salt head group with the cationic species depends on the pKa of the corresponding bile acids. Bile salts of conjugated head groups were found to be better candidates to capture the cationic species as compared to that of bile salts having non-conjugated head groups. The hydrophobic interaction dominates as aggregation of bile salts take place at higher concentration and results in the entrapment of the neutral species. The rotational relaxation of cationic species of ellipticine was found to be higher in di-hydroxy bile salt aggregates compared to that in tri-hydroxy bile salt aggregates. However, rotational relaxation of rotational relaxation of neutral ellipticine species is independent of the nature of bile salts.

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