Dynamics of Bound Dyes in a Nonpolymeric Aqueous Gel Derived from a Tripodal Bile Salt

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Rotational dynamics of polarity sensitive fluorescent dyes (ANS and DPH) in a nonpolymeric aqueous gel derived from tripodal cholamide **1** was studied using ultrafast time-resolved fluorescence technique. Results were compared with that of naturally occurring di- and trihydroxy bile salts. ANS in the gel showed two rotational correlation time (ϕ) components, 13.2 ns (bound to the hydrophobic region of the gel) and 1.0 ns (free aqueous ANS), whereas DPH showed only one component (4.8 ns). In the sol state, faster rotational motion was observed, both for ANS and DPH. Our data revealed that dyes get encapsulated more tightly in the gel network when compared to the micellar aggregates. ANS has more restrained rotation compared to DPH. This was attributed to the interaction of the sulfonate group of ANS with water molecules and hydrophilic parts of the gelator molecule. No restricted rotation was observed for DPH in the gel state unlike when it is in the gel phase of lipid bilayer.

1. Introduction

Bile salts conjugated with glycine and taurine participate in a number of important physiological processes such as in the emulsification of neutral fats and solubilization of cholesterol.[1] In aqueous environments, bile salts aggregate to form micelles,[2] and gels [3] depending on the number and position of the hydroxyl groups, concentration, pH, ionic strength, etc. Unlike conventional surfactant molecules, bile salts possess a rigid steroid backbone having polar hydroxyl groups on the concave α face and methyl groups on the convex β face (Chart 1). This arrangement creates a unique facial amphiphilicity for this class of molecules, enabling them to aggregate in aqueous media. Aggregation of bile salts in aqueous solution is largely driven by hydrophobic association of apolar β faces of steroid backbones, whereas further aggregation occurs through hydrogen bonding interactions.[1−3] The aggregation number of these bile salt micelles ranges from 2 to 10 for globular primary aggregates which increases as a function of bile salt concentration and added salt concentration to form larger rodlike secondary aggregates which was confirmed by small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANX).[2e−g] Although these micellar aggregates have been of considerable interest during the past several decades, there is very little information available on the hydrogels[4] derived from bile salts.[3] We have embarked on a long-term project to evaluate and understand the aggregation behavior of bile acid analogues in water[5] and have recently reported the outstanding gelling ability of a novel tripodal bile salt (**1**.H$^+$) in aqueous fluids and demonstrated the existence of hydrophobic pockets in the gel network.[5a] To utilize these gels for possible applications involving gel-encapsulated dyes/drugs/other bioactive molecules etc., it is important to understand the nature of these hydrophobic pockets, especially the dynamic behavior of a bound molecule in such a constrained environment. Herein, we report the first study of the dynamics of fluorescent dyes bound in the gel network of nonpolymeric[6] aqueous gel derived from **1**.

2. Experimental Section

2.1. Materials. 8-Anilino-1-naphthalene sulfonic acid (ANS, Fluka), 1,6-diphenylhexatriene (DPH, Aldrich), cholic acid (Aldrich), and sodium deoxycholate monohydrate (Lancaster) were more then 98% pure and used as received. Tripodal cholic acid derivative, nonahydroxy (**1**), was synthesized and purified using a previously reported procedure.[5a] All solvents were distilled prior to use.

2.2. Sample Preparation. Gelator **1** was dissolved in an appropriate quantity of acetic acid (AcOH) to which aq. ANS was added. Final concentrations of **1**, AcOH, and ANS were 5.25 mM, 20% (v/v), and 10 μM, respectively. It formed a thermoreversible gel with $T_{gel\rightarrow sol} = 65$ °C. A 0.6 mM solution of **1** in 20% AcOH−water formed only aggregates in solution which did not gel. Micellar solutions were prepared by dissolving sodium deoxycholate (NaDC, **2)** or sodium cholate (NaC, **3**) in water containing ANS. Concentration of **2** or **3** was 24 mM. The pH's of the resultant solutions were adjusted to 8.5 with NaOH. For all experiments, concentrations of ANS and DPH were 10 μM and 0.8 μM, respectively.

2.3. Measurements and Analysis. Steady-state fluorescence spectra were recorded on Perkin-Elmer LS 50B luminescence spectrometer. All data were collected for the samples under equilibrium conditions (i.e., when no change in either steady-state fluorescence intensity or fluorescence anisotropy was observed). Time-resolved fluorescence decay measurements of the samples were made using a high repetition rate frequency tripled picosecond Ti:sapphire laser (Spectra Physics) coupled

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CHART 1

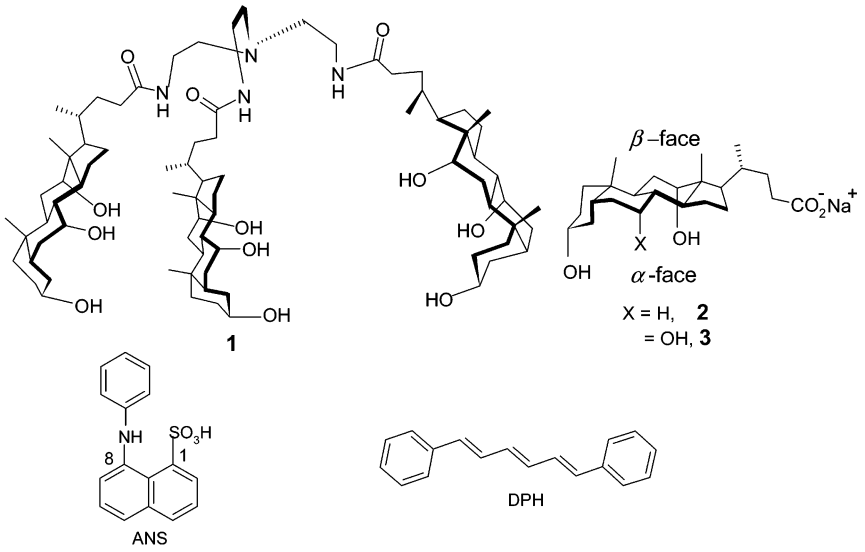

TABLE 1: Typical Parameters Associated with the Fluorescence Anisotropy Decay of ANS and DPH (in the Parenthesis)

probes	samples	temp	rotational correlation time[a] (in ns) ϕ_1	ϕ_2	amplitudes β_1	β_2	steady-state anisotropy (r_{ss})	χ^2
ANS (DPH)	gel from **1**	25 °C	13.2 (4.8)	1.0	0.60	0.40	0.05 (0.13)	1.20 (1.15)
	sol from **1**	75 °C	2.0 (1.3)	0.3	0.40	0.60	0.03 (0.07)	1.04 (1.01)
	aggregate **1**	25 °C	5.7 (3.0)	0.3	0.45	0.55	0.04 (0.08)	1.13 (1.45)
	aggregate **1**	75 °C	1.0 (1.0)	0.1	0.13	0.87	0.01 (0.07)	1.44 (1.12)
	micelle **2**	25 °C	6.0 (3.5)	0.3	0.39	0.61	0.05 (0.09)	1.29 (1.12)
	micelle **2**	75 °C	0.9 (1.0)	0.1	0.27	0.73	0.02 (0.06)	1.38 (1.20)
	micelle **3**	25 °C	1.7 (2.0)	0.3	0.70	0.30	0.04 (0.08)	1.21 (1.05)

[a] For micellar solutions maximum errors were (for ANS) ±0.4 and ± 0.1 ns, for ϕ_1 and ϕ_2 respectively, and for DPH: ±0.2 ns. In the gel (for ANS): $\phi_1 = 13.2 \pm 0.3$ ns and $\phi_2 = 1.0 \pm 0.2$ ns; (for DPH) $\phi = 4.8 \pm 0.4$ ns. ANS in 20% AcOH−water has ϕ of 90 ± 10 ps at 25 °C and 50 ± 15 ps at 75 °C. Data reported in the Table 1 are those with minimum χ^2 and with randomness of the plot of residuals vs channel.

to a time-correlated single photon counting spectrometer.[7] Samples were excited at 307 nm, and emissions were measured at 490 nm. For all of the measurements, at least 10K counts were collected at the peak. Steady-state anisotropy was determined as described in ref 7. For time-resolved anisotropy measurements, the emission data were collected at 0° and 90°with respect to the excitation polarization, and the time-resolved anisotropy decays were analyzed on the basis of the following model:

$$I_{II}(t) = I(t)[1 + 2r(t)]/3 \quad (1)$$

$$I_{\perp}(t) = I(t)[1 - r(t)]/3 \quad (2)$$

$$r(t) = r_o \sum_{j=1}^{n} \beta_j \exp(-t/\phi_j), \quad \text{where } j = 1 \text{ or } 2 \quad (3)$$

The *G* factor of the detection optical asembly was estimated by using a solution of fluorescein in water. $I(t)$ is the fluorescence intensity collected at magic angle (54.7°) at time t, r_0 is the initial anisotropy, and β_j is the amplitude associated with the jth rotational correlation time (ϕ_j). For details of experimental methods and data analysis, please see ref 7.

2.4. Dynamic Light Scattering Measurements. Dynamic light scattering experiments were performed on a DynaPro-MS800 instrument (Protein Solution Inc., VA) which monitors the scattered light at 90°. At least 20 measurements each of 10 s were collected. NaC solution (24 mM) was filtered through 0.02 μ filteres followed by centrifugation at 12 000 rpm for 20 min. "Regularization" software provided with the intrument was used to analyzed the data to obtain hydrodynamic radius. A solution of bovine serum albumin (3 nm) was used as a standard.

3. Results and Discussion

The onset of the gelation process was characterized by a sharp increase in the steady-state fluorescence intensity of hydrophobic fluorescent probes such as ANS[5a] and DPH. In the present work, all experiments were carried out in 20% AcOH−water, in which the critical gel concentration of **1** is 0.1% w/v (0.75 mM). Using steady-state fluorescence titration, we have studied the interaction of ANS with increasing amounts of **1** to monitor the onset of the aggregation process (not shown). At the concentration range of 0.3−0.4 mM, an increase in the fluorescence intensity was observed possibly because of the formation of primary aggregates. No gelation occurred below 0.75 mM, and near this concentration, further increase in the fluorescence intensity was observed, suggesting secondary aggregation processes which lead to gelation. A similar mechanism was proposed for other bile salts.[1−3]

The time-resolved decay of the fluorescence anisotropy [$r(t)$] of the organic dye molecule is directly related to the reorientation dynamics of excited molecules and hence best suited for the investigation of local molecular dynamics near the binding site.[7] We have used picosecond laser to probe the local structure and dynamics of the aggregates and gel. The decay of the fluorescence anisotropy of ANS in the gel (Figure 1B) showed two rotational correlation time (ϕ) components (Table 1): 13.2 ns (60%) and 1.0 ns (40%). ANS partitioned in the hydrophobic

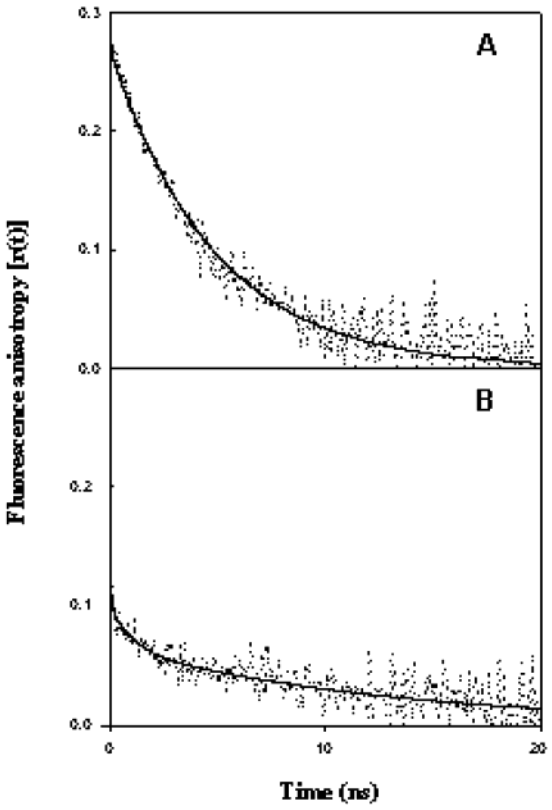

Figure 1. Fluorescence anisotropy decay of DPH (A) and ANS (B) in the gel-network produced from tripodal cholamide **1** at 25 °C. Solid lines represent the single-exponential fit for A and biexponential fit for B (eq 3). A: Rotational correlation time (ϕ) = 4.8 ns; $\chi^2 = 1.15$. B: Rotational correlation times: $\phi_{slow} = 13.2$ ns (60%, bound ANS) and $\phi_{fast} = 1.0$ ns (40%, free ANS) $\chi^2 = 1.20$.

region of the gel is represented by the longer correlation time, whereas ANS in the aqueous phase surrounding the gel network is represented by the shorter component. This short ϕ is higher than the ϕ of ANS in a neat aqueous medium ($\phi \approx 0.1$ ns) which is expected due to partial immobilization of water molecules in the aqueous phase the gel fibers. Therefore, in the gel-state, in both hydrophobic and solvent accessible regions, ANS has a slower tumbling motion. In contrast to this picture in the gel, ANS showed faster rotational motion in the sol at 75 °C. Again, two rotational correlation times were observed, 2.0 ns (40% amplitude) and 0.3 ns (60% amplitude), suggesting two populations of ANS, with the short ϕ representing free, aqueous ANS. The longer one is either due to ANS bound to the hydrophobic part of the gelator molecule or its small aggregates present at this temperature. The difference in ϕ's in the gel and in the sol may be due to either a difference in the microviscosity felt by ANS or difference in the molecular volume of ANS-bile salt complex. According to the Stokes−Einstein equation

$$\phi = \eta V/kT \qquad (4)$$

(where η 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 or V).[8] From our data, it is also possible to estimate the microviscosity felt by the "aqueous" population of ANS. ANS in 20% AcOH−water has a rotational correlation time of about 90 ps at 25 °C (viscosity of this solution is about 1.2 cP at 25 °C). Hence the observed $\phi_2 \approx 1.0$ ns, would correspond to a viscosity of about 13 cP in the aqueous phase.

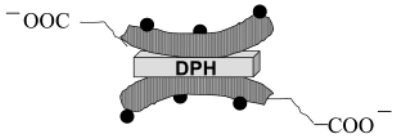

Figure 2. Proposed structure (after Small's model[2a,2b]) of the lipophilic dye solubilization in the dimeric miceller aggregate formed by **3** in water at pH 8.5.

ANS in the micellar aggregate of NaDC (**2**) in water showed two correlation times: 6 ns, which indicated ANS bound to the pocket created by the β faces several steroid units,[9] and 0.3 ns (free ANS). Our data reveal that the situation is quite similar ($\phi_1 = 5.7$ ns) in the case of "aggregates" formed by the tripodal bile salt **1** at 0.6 mM concentration (Table 1). ANS bound to a typical dimeric aggregate[9] from NaC (**3**) showed much faster rotational motion ($\phi_1 = 1.7$ ns) compared to that in more complex aggregates from both NaDC (dihydroxy) and the tripodal bile salt (nonahydroxy).

Interestingly, fluorescence anisotropy decay of DPH showed only one component for all of the samples studied by us (Table 1). Figure 2 gives a cartoon representation indicting the solubilization of DPH in the dimeric micellar aggregate of NaC.[9] The rotational correlation time of DPH in the simplest miceller system (in NaC) was about 2 ns. In the gel phase (Figure 1A), ϕ of DPH was 4.8 ns, whereas in the sol phase, ϕ was 1.3 ns, significantly larger than the value expected ($\leq$0.2 ns) for DPH in a solution of low viscosity. This would indicate that DPH, being more hydrophobic than ANS, would always be complexed to the hydrophobic regions of the aggregates.

Therefore, partitioning of a dye between the hydrophobic region and the solvent accessible region would depend not only on the nature of the pocket but also on the nature of the dye molecules. From our data, it is clear that the gel network offers somewhat more compact hydrophobic pockets (in terms of the microviscosity felt by the probes) compared to bile salt micelles. It is interesting to note that ANS has longer correlation time compared to DPH in the gel state (13.2 and 4.8 ns, respectively). This is not surprising because of the fact that the hydrophilic sulfonate group in the ANS molecule is likely to straddle at the interface, enabling it to hydrogen bond to solvent molecules and/or the hydrophilic part of **1**. This in turn would keep ANS more anchored causing damping of the rotational motion, and hence, an increase in the rotational correlation time is observed. It should be pointed out that this information can be derived only from the time-resolved anisotropy, because the steady-state anisotropy (r_{ss}) data (Table 1) would have been erroneously interpreted to higher mobility of ANS (compared to DPH). This is because of the fact that r_{ss} of ANS is complicated by the changing lifetimes and the presence of short rotational correlation time in aqueous phase.

Another significant observation is that there is no residual anisotropy ($r_{infinity}$) of the probes in the gel, indicating the absence of restricted rotation. This is unlike the case of linear DPH which cannot make an unrestricted 90° rotation in the gel phase of a lipid bilayer. We therefore postulate that the rigidity of the interior structure of the gel is between that of lipid membranes and miceller aggregates of bile salts.

In light of a newer interepretation of time-resolved anisotropy decay of a probe in silica sol−gels,[6c] it is necessary to address the issue of local (rotation of probe within the aggregate) and/or global dynamics (rotation of the whole aggregate). Our dynamic light scattering experiments[10] revealed that the mean hydrodynamic radius ($\bar{R}_h$) of NaC is 12 Å (1.2 nm), which is in good agreement with the size reported earlier by others for

trihydroxy bile salt micelles.[2d] The global rotaional correlation time of a spherical micelle (ϕ_m) is given by

$$\phi_m = 4\pi R_h^3 \eta/3kT \quad (5)$$

Using $\eta = 0.9$ cP for water at 25 °C the ϕ_m of NaC is estimated to be 0.1.6 ns which is close to the data obtained for both DPH and ANS (1.7−2.0 ns). Therefore, it seems likely that our studies of these *micellar aggregates* report the global rotation of a micelle or both global rotation and local rotation occur in the same time scale. However, the situation in the gel state is different and much more complicated. A gel is formed by the linear one-dimensional growth of a supramolecular assembly which leads to the formation of an entangled network. Our cryotransmission electron microscope (TEM) studies have suggested dimension of these fibers to be of the order of 10 nm.[5c] For a large micelle like Triton X-100 ($R_h = 4.3$ nm), $\phi_m = 72$ ns.[7a] So, the rotational correlation time of ANS in the gel (13.2 ns) would not represent the global rotation of the aggregate which is clearly much larger compared to Triton X-100 micelles. Also very different dynamics of ANS and DPH in the gel state supports this interpretation since according to "global rotation" model rotational diffusion should be largely independent of the nature of the bound dye and should depend only upon size of the aggregates. It is possible that increase in the rotational correlation time is due to smaller probe-containing microparticles (subunits of gel fibers) in the gel wherein the particles rotate within the gel matrix. At the same time, the effect due to microviscosity cannot be ruled out. Therefore, the rotational dynamics of ANS in gel state is controlled by three factors: (*i*) the microviscosity felt by the probe (which is much higher compared to the 13 cP estimated for ANS in the aqueous phase surrounding the gel network), (*ii*) the volume of the rotating unit, and (*iii*) additional damping interactions coming from the sulfonate group. In contrast, the dynamics of DPH is controlled by first two factors. Assuming only the first factor contributes significantly, microviscosity of hydrophobic pockets in the gel phase estimated to be 120 ± 20 cP.[11]

4. Conclusions

In conclusion, we have measured, for the first time, the dynamics of organic dyes partitioned between hydrophobic pockets and the bulk aqueous phase in a nonpolymeric hydrogel. We have also demonstrated that dynamics in the gel-state would depend on the chemical nature of a dye. We believe that the intriguing information about the dynamics of the dye molecules intercalated in the gel network would be of material and biological interest. Systematic studies aimed at evaluating the influence of the bile acid structure and particle growth on the rotational dynamics of the bound dyes are currently in progress and will be reported elsewhere.

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References and Notes

(1) (a) Danielsson, H. In *The Bile Acids: Chemistry, Physiology and Metabolism*; Nair, P. P., Kritchevsky, D., Ed.; Plenum Press: New York, 1973; Vol. 2, pp 1−32. (b) Carey, M. C. In *Sterols and Bile Acids*; Danielsson, H., Sjövall, J., Ed.; Elsevier: Amsterdam, 1985; pp 354−425. (c) Hofmann, A. F. In *Bile Acids and Hepatobiliary Disease*; Northfield, T., Zentler-Munro, P. L., Jazrawi, R. P., Ed.; Kluwer Academic Publishers: Boston, 1999; pp 303−332 and references therein. (d) Carey, M. C. In *Phospholipids and Atherosclerosis*; Avogaro, P., Ed.; Raven Press: New York, 1983; pp 33−63.

(2) (a) Small, D. M. In *The Bile Acids: Chemistry, Physiology and Metabolism*; Nair, P. P., Kritchevsky, D., Ed.; Plenum Press: New York, 1971; Vol. 1, pp 249−356. (b) Small, D. M. *Adv. Chem. Ser.* **1968**, *84*, 31. (c) Kratohvil, J. P. *Adv. Colloid Interface Sci.* **1986**, *26*, 131. (d) Mazer, N. A.; Carey, M. C.; Kwasnick, R. F.; Benedek, G. B. *Biochemistry* **1979**, *18*, 3064. (e) Esposito, G.; Giglio, E.; Pavel, N. V.; Zanobi, A. *J. Phys. Chem.* **1987**, *91*, 356. (f) Lopez, F.; Samseth, J.; Mortensen, K.; Rosenqvist, E.; Rouch, J. *Langmuir*, **1996**, *12*, 6188. (g) Hjelm, R. P.; Schteingert, C. D.; Hofman, A. F.; Thiagrajan, P. *J. Phys. Chem. B* **2000**, *104*, 197. (h) Coello, A.; Meijide, F.; Rodríguez Núñez, E.; Vázquez Tato, J. *J. Pharm. Sci.* **1996**, *85*, 9 and references therein.

(3) (a) Rich, A.; Blow, D. M. *Nature* **1958**, *182*, 423. (b) Blow, D. M.; Rich, A. *J. Am. Chem. Soc.* **1960**, *82*, 3566. (c) Igimi, H.; Carey, M. *J. Lipid. Res.* **1980**, *21*, 72. (d) Terech, P.; Smith, W. G.; Weiss, R. G. *J. Chem. Soc., Faraday Trans.* **1996,** *92*, 3157.

(4) For recent reports on hydrogelators, see: (a) Bhattacharya, S.; Acharya, S. N. G. *Chem. Mater.* **1999**, *11*, 3504. (b) Estroff, L. A.; Hamilton, A. D. *Angew. Chem., Int. Ed.* **2000**, *39*, 3447. (c) Menger, F. M.; Caran, K. L. *J. Am. Chem. Soc.* **2000**, *122*, 11679. (e) Kobayashi, H.; Friggeri, A.; Koumoto, K.; Amaike, M.; Shinkai, S.; Reinhoudt, D. N. *Org. Lett.* **2002**, *4* (9), 1423−1426.

(5) (a) Maitra, U.; Mukhopadhyay, S.; Sarkar, A.; Rao, P.; Indi, S. S. *Angew. Chem., Int. Ed.* **2001**, *40*, 2281. (b) Sangeetha, N. M.; Balasubramanian, R.; Maitra, U.; Ghosh, S.; Raju, A. R. *Langmuir*, **2002**, *18*, 7154. (c) Mukhopadhyay, S.; Maitra, U.; Talmon, I. Unpublished results.

(6) For applications of ultrafast spectroscopy in polymeric sol−gel systems, please see: (a) Narang, U.; Wang, R.; Prasad, P. N.; Bright, F. V. *J. Phys. Chem.* **1994**, *98*, 17. (b) Birch, D. J. S.; Geddes, C. D. *Phys. Rev. E.* **2000**, *62*, 2977. (c) Geddes, C. D.; Karolin, J.; Birch, D. J. S. *J. Phys. Chem. B* **2002**, *106*, 3835.

(7) (a) Maiti, N. C.; Krishna, M. M. G.; Britto, P. J.; Periasamy, N. *J. Phys. Chem. B* **1997**, *101*, 11051. (b) Lakshmikanth, G. S.; Krishnamoorthy, G. *Biophys. J.* **1999**, *77*, 1100. (c) Lakshmikanth, G. S.; Sridevi, K.; Krishnamoorthy, G.; Udgaonkar, J. B. *Nat. Struct. Biol.* **2001**, *8*, 799.

(8) Only increase in the temperature from 25 to 75 °C would have modified ϕ_1 from 13.2 to 11.3 ns (obtained from the eq 4). So, clearly the change in ϕ's are result of change in η or V or both.

(9) NaDC forms higher aggregates instead of only dimers, whereas NaC forms largely dimeric aggregates in the absence of added salt (ref 2a,b). However, still the aggregation number of bile salts is a matter of debate (ref 2h).

(10) The experimental conditions were same as that for time-resolved anisotropy. [NaC] = 24 mM, pH = 8.5, temperature = 25 °C. So, we were able to compare the light scattering data with the anisotropy decay. Primary micelles from bile salts were previously reported to be nearly spherical (ref 2).

(11) It was estimated from eq 4 using the effective volume of DPH in paraffin oil (which ranges from 15×10^{-23} to 18×10^{-23} cm^3) reported earlier: Kawato, S.; Kinosita, K.; Ikegami, A. *Biochemistry* **1977**, *16*, 2319. We assumed effective volume in the hydrophobic region of the aggregate remains same as that in liquid paraffin.