Self Association of Bile Salts in Aqueous Medium - A Spectroscopic Investigation using Diphenylhexatriene

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CERTIFICATE

This is to certify that the dissertation entitled **"Self Association of Bile Salts in Aqueous Medium – A Spectroscopic Investigation using Diphenylhexatriene"** being submitted by Swapnadip Roy to the Department of Chemistry, National Institute of Technology, Rourkela, Orissa, for the award of the degree of Master of Science is a record of bonafide research carried out by him under my supervision and guidance. To the best of my knowledge, the matter embodied in the dissertation has not been submitted to any other University / Institute for the award of any Degree or Diploma.

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1. Introduction

Concept of Surfactants:

All soluble substances can be divided into two groups according to their ability to be adsorbed at the liquid-air interface: Surface active substances and Surface-inactive substances [1-3].

Surface –active substances:

These substances which are also called as surfactants are capable of accumulating in the surface layer. Surfactants must possess surface tension which is less than that of a solvent; otherwise the accumulation of a substance in the surface layer is thermodynamically disadvantageous. Their solubility must be comparatively small or they would tend to sink from the surface into the depth of a liquid. In other words, the interaction between the molecules of a surfactant and a solvent is always less than that between the molecules of the solvent. As a result of the accumulation of surfactant molecules on the solvent surface, which poorly interact with one another, the surface tension decreases hence the molecules are termed as surface-active molecules [2-3].

Surface –inactive substances:

These substances tend to go away from the liquid surface in the volume. Surface – inactive substances have surface tension which is greater than that of a solvent, otherwise, they would spontaneously accumulate in the surface layer. They usually possess high solubility, which favors their tendency to submerge into the volume of a liquid. In other words, the interaction between the molecules of a surface-inactive

substance and of a solvent is always greater than that of the intermolecular interactions of the solvent [2-3].

Critical Concentration of Micelle Formation:

All surfactants are characterized by low solubility and ability to reduce surface and interfacial tensions in dilute solutions. But at a certain concentration, known as the critical concentration of micelle formation or critical micelle concentration (CMC), molecular aggregates or micelles are formed in the solution. Thus, Critical Micelle Concentration (CMC) of a surfactant is defined as the surfactant concentration at which micelles first appear in the solution [1-2].

Bio-surfactants:

Bio-surfactants are biologically produced amphiphilic molecules, which when dispersed in water tend to organize themselves spontaneously in a definite fashion such as micelles, bilayer, vesicles, emulsions or micro-emulsions *etc.* depending on their concentrations, molecular structure and experimental conditions. Most of the bio-surfactants are either anionic or neutral amphiphilic molecules [3]. Examples of bio-surfactants are glycosides, phospholipids, fatty acids, neutral lipids, bile salts etc [3].

Bile Salts as Bio-Surfactants :

Bile salts are regarded as the most important “bio-surfactants” because of their unusual solubilizing and emulsifying capacity. The main function of bile salt is to solubilize dietary lipids, cholesterol and fat soluble vitamins in the gastrointestinal tract. The solubilisation quality of bile salts is due to their micellisation capacity. In view of their unique molecular structure, they do not behave like conventional surfactants that contain

a clear-cut polarity gradient between the hydrophilic and hydrophobic parts. They show distinct behavior with respect to self–association and molecular solubilisation [4,5]. Behavior of bile salts in aqueous medium has been a subject of much interest and controversy for a long time. Bile Salts are biocompatible and biodegradable and hence they are very safe and effective vehicle for medical application [4-6]. That is why bile salts are being extensively used as delivery systems for drugs, vitamins etc [5-9].

Structure of Bile Salts:

Bile salts are synthesized from cholesterol in the liver. These are derivative of Cholanic acid that possesses a four-ring steroid nucleus and a five-carbon side chain terminating in a carboxylic acid. The arrangement of the rings is such that all the hydrophilic parts such as the hydroxyl groups and the carboxylic acid side arm positioned on one plane and the hydrophobic parts remain on the other, thus bile salts have a planar polarity. Because of the presence of amphiphilic nature and planar polarity bile salts tend to aggregate in water. In the following figure the A/B rings are *cis* and hydrogen on C5 is β (below the plane of the molecule) [5].

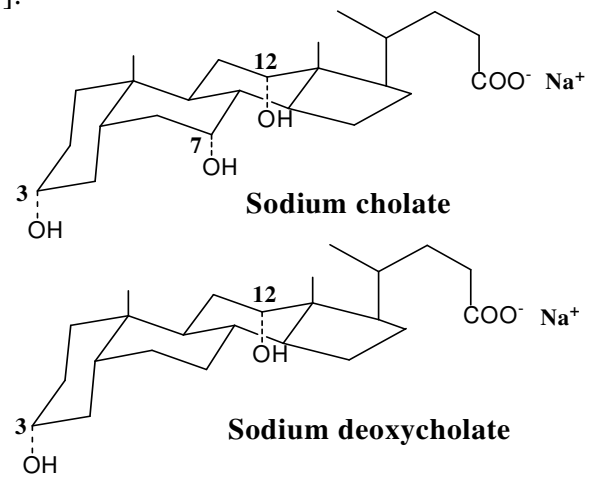

Fig.1 Molecular structures of bile salts, sodium cholate (trihydroxy) and sodium deoxycholate (dihydroxy)

Substitution of hydroxyl groups into the steroid nucleus yields mono-, di- and tri-hydroxy bile salts. These hydroxyl groups can be accommodated either above (α) or below (β) the plane of the molecule and can be located at positions C3, C6, C7 or C12, thus giving rise to a number of bile salts [5]. Besides, conjugation of the carboxyl side chain with taurine and glycine or other amino acids increases the possibilities of many more bile salts (Fig.1).

Amphiphilic Nature of Bile Salts :

The cis nature of A/B rings of the steroidal nucleus make the nucleus somewhat kinked and, with its five-carbon chain also slightly angled, the molecule resembles an overturned flattened teaspoon with a short handle. The hydroxyl groups lie on the same side of the molecule on the face of the spoon, giving rise to a hydrophilic domain of a size depending on the number of the hydroxyl groups present. The carboxylate or conjugate anion of the side-chain is angled on the same side of the molecule as the ring hydroxyls, thus playing the role of handle in the spoon. The back of the spoon, which includes the unsubstituted regions of the hydrocarbon rings, together with the methyl groups of the hydrocarbon rings and of the side-chain, forms an extensive hydrophobic domain. The presence of these hydrophilic and hydrophobic domains imparts bile salts the desired amphiphilic nature. The number of hydroxyl groups and their orientations, the length and polarity of the side-chain and any conjugate on the ring hydroxyls contribute greatly to the hydrophilic**:**hydrophobic balance of bile salt molecules and decides their solubility in aqueous medium. In contrast to most conventional amphiphiles derived from aliphatic hydrocarbons, which tend to be rod-shaped molecules, bile salts are somewhat flattened and occupy a relatively large surface area. Due to the presence of planar polarity, bile

salts line up at an oil-water or air-water interface with the hydrophilic domain orientated into the aqueous environment [10].

Micellisation of Bile Salt

Extensive work has been carried out by many scientists on the aggregation behavior of bile salts in aqueous medium after the pioneering work of McBain and co-workers in 1940's [10-17]. The process of micellisation of bile salts is less well defined and poorly understood as compared to the conventional alkyl chain detergents where the aggregation proceeds with their hydrocarbon chains forming a core and the polar head groups on the surface. In fact the mechanism of formation of bile salts micelles is still a topic of controversy [10,18-20] and there are two most debated models (I) Small's models of primary and secondary micellisation based on High-Resolution ^{1}H Nuclear Magnetic Resonence (NMR) Technique [10] and (II) Oakenfull and Fisher's models based on Conductance Measurements of Bile Salts in water [18-20].

(I) Small's Model:

According to D.M Small [10], Bile Salts form two types of aggregation (i) Primary micelle (ii) Secondary micelle. Former consists of two to nine monomers which are held by hydrophobic interaction. And the larger aggregates are formed by repeated aggregation of the primary micelles, which are held mostly by hydrophilic interactions. Primary micelles are supposed to be globular in shape, whereas the secondary micelle are regularly globular having oblate ellipsoidal structure.

(II) Oakenfull and Fisher's Model:

According to Oakenfull and Fisher [18-20] the first stage of aggregation is mainly due to the formation of dimer having maximum number of hydrogen bonds and charged

carboxylic groups, the dimers are supposed to be stabilized by the hydrogen bonding and hydrophobic interaction. The back-to-back hydrophobic interaction between the dimers helps formation of layer aggregates of bile salts. This is in tune with the suggestion of D. M. Small for the formation of primary micelle. This model also suggests that tetramers or higher units of secondary micelles are formed by rod-like stack of dimers.

Different techniques used for study of micellisaiton

Micellisation process often brings about several changes in the system of interest, which can be related to appreciable alterations in phenomena such as light scattering, surface tension, viscosity and solubilisation of small organic molecules *etc* [14]. These changes can be monitored as a function of concentration of the surfactants giving rise to several analytical techniques that can be employed for the investigation of micelles formation. NMR [10,21], Spectrophotometry [13] and Fluorescence [13,14,21-24] are the most useful techniques used for the same.

Fluorescence technique is a powerful tool for the investigation of structural and dynamic aspects of matter and living systems at a molecular or supramolecular level [25]. The advantages of using fluorescence technique are because of its sensitivity, suitable time-scale, non-invasive nature and minimal perturbation. Fluorescent molecular probes have received a great deal of attention for the study of organised media and the number of chromophores capable of serving as fluorescent probes is constantly increasing [25].

Recently Subuddhi et. al [14,15] have used three conceptually different fluorophores (coumarin1 (a polarity sensitive probe showing ICT character), nile blue A (a cationic fluorophore) and 1,6-diphenylhexatriene (a neutral hydrophobic fluorophore)) for the

study of micellisation of NaDC and NaC in aqueous medium. Among the three 1,6-diphenylhexatriene (DPH) was found to be most sensitive and provided important information about the micellisation process [14,15]. This study revealed that for the same concentration of DPH under similar experimental conditions the fluorescence intensity in NaDC is always less than that in NaC micelles. This implies that the microenvironment of micelles formed by NaDC is more hydrophobic than that of NaC bile salts. In order to verify whether this observation holds good for all dihydroxy and trihydroxy bile salts we set our aim of this present work.

Aim of the Present Work:

So the specific aims of the present work are to find out,

- ✓ Whether the intensity difference observed between NaC and NaDC is a general phenomenon i.e. Do all cholate salts behaves similar to NaC and all deoxycholate salts behaves similar to NaDC? And if yes then
- ✓ What is the reason behind this difference in behavior of DPH in cholate and deoxycholate?

2. Materials and Methods

Materials:

Fluorophore Used

1,6-diplenylhexatrine (DPH) was purchased from Sigma Chemical Company (USA).

Medium Components: The bile salts, sodium cholate (NaC), sodium deoxycholate (NaDC) were obtained from SRL India and sodium taurocholate (NaTC), sodium

taurodeoxycholate (NaTDC) were obtained from Sigma Chemical Company (Bangalore, India).

Solvents: Deionised water was used for all the experiments. Ethanol used was of spectrograde quality and used as received.

Instrumentation: The absorption spectra were recorded using ***Hitachi Spectrofluorimeter (FL-7000)*** and the emission spectra were recorded using ***Shimadzu Spectrophotometer (UV-2450).***

Methods :

Preparation of Bile Salts Solution: For the bile salts studies, the stock solution of NaDC, NaC, NaTDC and NaTC were prepared in Millipore water and the experimental solutions were prepared by subsequent dilutions from the stock. Fresh solutions of bile salts were prepared every time to avoid the problem of aging. The sample containing DPH was prepared by adding a small amount (~μL) of an ethanolic solution of DPH into the experimental solution of the bile salts.

3. Results and Discussions

In order to check whether the intensity difference that was observed between NaDC and NaC [15] holds good for all dihydroxy and trihydroxy bile salts, fluorescence study was carried out with sodium taurocholate (NaTC) and sodium taurodeoxycholate (NaTDC) which are very similar in structure with sodium cholate and sodium deoxycholate. The emission spectra of DPH in NaTDC and NaTC at various concentrations of respective bile salts in aqueous medium are shown in Fig. 2 (A) and (B), respectively.

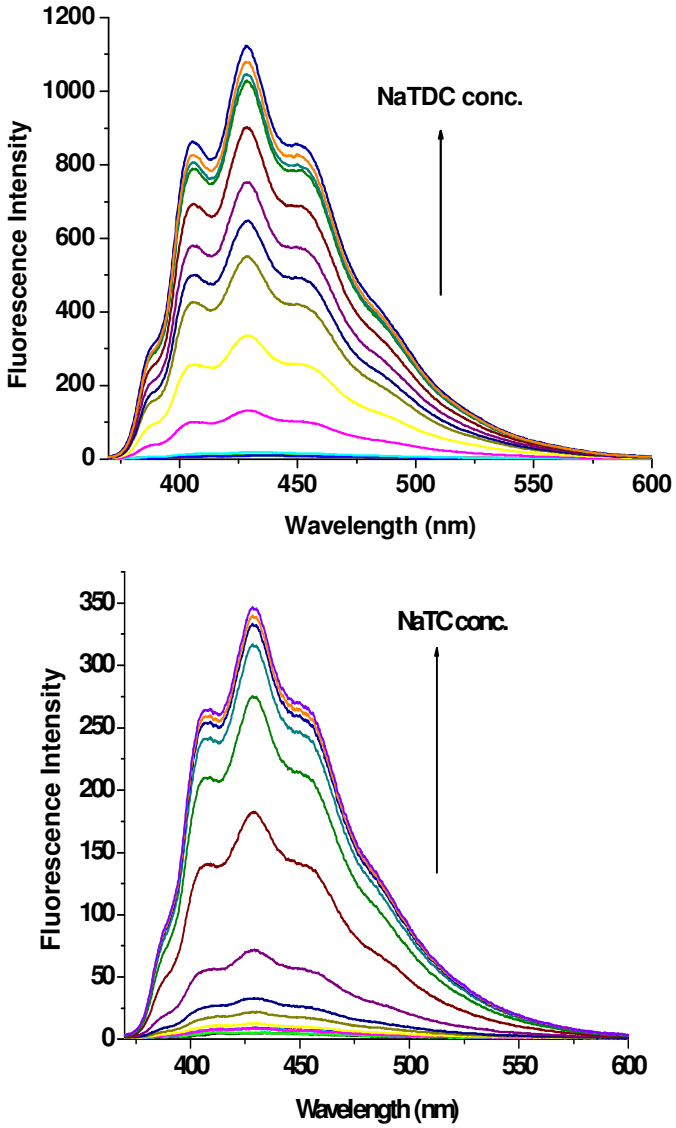

Fig.2 Emission spectra of DPH in **(A)** NaTDC and **(B)** NaTC in water with increasing concentration of NaTC,

From the above two plots it is clear that the maximum fluorescence intensity for NaTDC is around 1200 and for NaTC it is around 350, this observation is more clear from The CMC values were also calculated from these plots and found to be ≈7 mM for NaTDC and ≈17 mM for NaTC, which agrees well with the reported values [26]. Now comparing the fluorescence intensities of DPH in NaTDC and NaTC it is very clear that the intensity of DPH in NaTDC is greater than that of NaC for the same concentration of DPH and

under similar experimental conditions. This behaviour is very similar to that observed earlier between NaDC and NaC. Thus it can be conclude that the intensity of DPH in cholate salts is always less than that of the corresponding deoxycholate salts i.e. all the dihydroxy bile salts behave very similar to that of NaDC and all trihydroxy bile salts behave like NaC.

In order to understand the reason behind the above observed phenomenon a series of spectrophotometric study of DPH in bile salts were carried out. But before going to the bile salts study it is desirable to know about the absorption behavior of DPH in water. For this absorption spectral studies were carried out for DPH in water. DPH being highly hydrophobic is practically water insoluble and needed minimum of 1% of ethanol (V/V) for complete solubilisation.

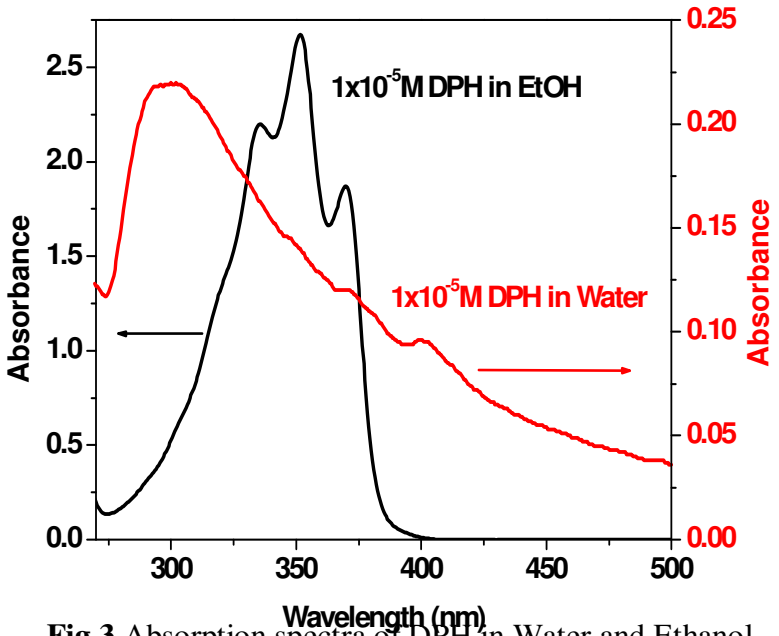

Fig.3 Absorption spectra of DPH in Water and Ethanol

Fig.3 represents the absorption spectra of DPH in water and ethanol. It is clearly seen that the absorption spectrum of DPH in water is broad and structureless. But it is known that *trans*-DPH shows absorption spectrum with three prominent vibrational bands [27],

which is clearly seen in the absorption spectrum of DPH in ethanol. There is no earlier report on absorption profile of *trans*-DPH in water. However there is a recent report [28] where the authors have synthesized *cis*-DPH and have carried out spectroscopic studies. To our surprise the absorption spectrum of *trans*-DPH in water matched exactly with that of *cis*-DPH. So we believe that the most preferred form for DPH in water is the *cis* form. But what drives this conversion from *trans*- to *cis*- form in water is not clearly understood.

In order to check the effect of bile salts on the absorption spectrum of DPH, spectrophotometric studies were carried out using bile salts NaDC and NaC,. Fig. 4 shows the absorption spectra of DPH at various concentrations of NaDC.

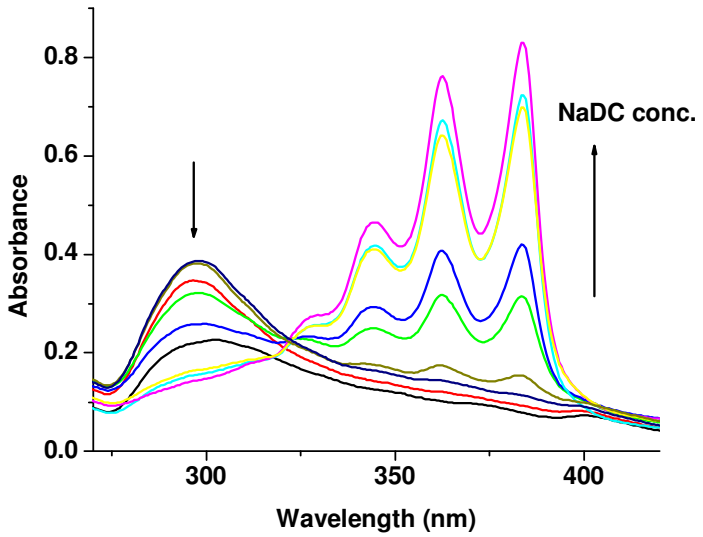

Fig. 4. Absorption spectra of DPH at different concentration of NaDC.

As seen in the figure with the increasing concentration of bile salt there is an increase in the structured absorption with a concomitant decrease in the broad and structureless absorption. Since the structured absorption is due to the trans form of DPH and the broad

structureless absorption band is due to the cis form of DPH, it can be inferred that with increasing the concentration of bile salts the *trans* form of DPH is increasing with a simultaneous decreasing in the *cis* form. A clear isobestic point observed around 323 nm indicates the presence of an equilibrium between *cis* and *trans* form.

Hence in water the most preferred form is *cis* but the *trans* form is predominant in NaDC. This implies that NaDC micelles provide a hydrophobic environment for DPH. In order to find what happens in NaC, similar studies were carried out in NaC. Fig 5 represents the absorption spectra of DPH at various concentrations of NaC.

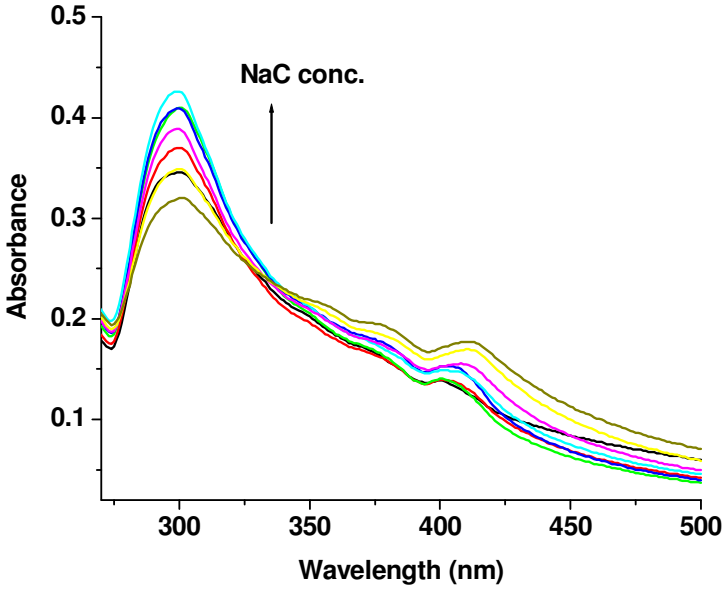

Fig.5. Absorption spectra of DPH at different concentration of NaC.

Unlike in case of NaDC, in NaC it is observed that there is not much difference between the absorption spectrum of DPH in absence and in presence of NaC except for a small increase in the structured absorption in presence of NaC. This implies that NaC provides a relatively hydrophilic environment for DPH in the micelle interior. Since it is already known that only the *trans* form of DPH is fluorescence active not the *cis* form and the

predominant form of DPH in NaC is *cis*-form and in NaDC it is in *trans*-form, this explains the fact that the fluorescence intensity of DPH in NaDC is always greater than that in NaC for the same concentration of DPH and under similar experimental conditions.

4. Conclusions

It is clear from the fluorescence study that the intensity of DPH in cholate micelles is less than that in corresponding deoxycholate micelles for a given concentration of DPH and under similar experimental conditions. From the intensity versus concentration graphs the CMC was calculated for NaTDC and NaTC to be 7 mM and 17 mM, respectively. The spectrophotometric study revealed that DPH exists in water is the *cis*-form. It is also inferred that Deoxycholate micelles provide a hydrophobic environment for DPH hence the preferred form for DPH in deoxycholate micelles is the *trans*-form and the intensity is high, whereas Cholate micelles provide a hydrophilic environment for DPH hence it exists in the *cis*-form and the fluorescence intensity is low. From the above studies it can be suggested that though the dihydroxy and trihydroxy bile salts are structurally very similar they aggregate differently in aqueous medium. The driving force for deoxycholate bile salt micellisation may be the *hydrophobic interaction*, whereas that for cholate bile salts in the *hydrogen bonding* or *hydrophilic interaction*s. Thus for the dexoycholate bile salts micellisation Small's model holds good whereas for cholate salts Oakenfull and Fisher model is more appropriate.

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