Interaction of a Cationic Dye "Nile Blue A" with Bile Salts

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CERTIFICATE

This is to certify that the dissertation entitled **"Interaction of a Cationic Dye "Nile Bule A" with Bile Salts"** being submitted by Nivedita Bose to the Department of Chemistry, National Institute of Technology, Rourkela, Odisha, for the award of the degree of Master of Science is a record of bonafide research carried out by her 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.

Rourkela
Date: 04-05-2011

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Date: 04-05-2011

(NIVEDITA BOSE)

CONTENTS

INTRODUCTION

1.1 Surface active agents:

Surface active substances or surfactants, as they are commonly known as; decrease the surface tension of a liquid [1]. These molecules have a strong tendency to migrate and accumulate at the air-water interface. Such migration is also thermodynamically favoured as the surfactant molecules have lower surface tension than the solvent. The interaction between the surfactant molecules and the solvents are less as compared to intermolecular solvent interaction and therefore the solubility is also less. Hence the surfactant molecules accumulate on the solvent surface thereby decreasing the surface tension [2].

1.1.1 Structure of surfactants:

Surfactants are generally amphiphilic organic molecules possessing polar hydrophilic head groups and nonpolar hydrophobic tail (fig.1.1). The polar portion exhibits a strong affinity for polar solvents, particularly water. Depending on the molecular structure and type, a balance between hydrophilicity and hydrophobicity exists in surfactant molecules. This is called the hydrophile– lipophile balance or HLB, which is important in categorizing surfactants as emulsifiers, detergents, etc. [3]. Surfactants having greater hydrophobicity are more surface-active and vice versa.

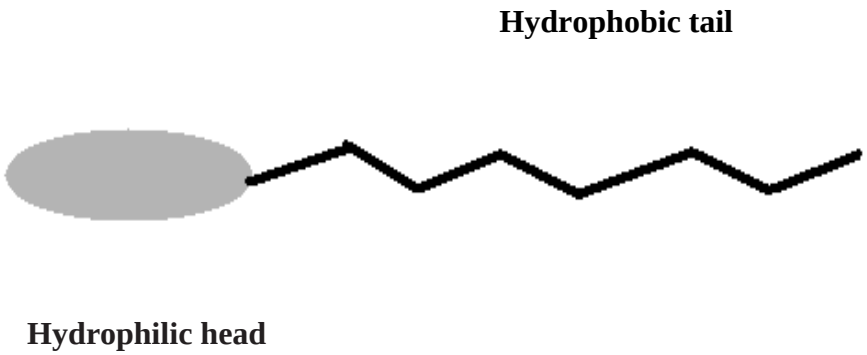


Fig.1.1 Structure of a conventional surfactant.

1.1.2 Critical micelle concentration (CMC):

The molecular shape of a conventional surfactant is conical *i.e.* its cross-section area is larger than the volume/length ratio; hence they tend to aggregate along curved surfaces forming micelles in aqueous solutions [4, 5]. Each surfactant has a certain critical concentration only above which micellisation takes place. This concentration is known as the critical micelle concentration or CMC [6]. According to Mukherjee and Mysels, the CMC value determines the industrial application and biological activity of detergents as well as some other interesting surfactant features like solute-solvent and solute-solute interactions.

1.1.3 Classification of surfactants:

Surfactants are classified primarily into three sub groups depending on the charge on the hydrophilic head group, (i) cationic like cetyl trimethylammonium bromide (CTAB), dioctadecyldimethylammonium bromide (DODAB), (ii) anionic like sodium lauryl sulphate (SDS), alkyl benzene sulphonates, and (iii) non-ionic surfactants like Tween 20, and Tx100. Surfactants can also be biologically synthesized and are called as bio-surfactants.

1.1.4 Bio-surfactants:

Bio-surfactants (BS) are class of biologically produced amphiphilic molecules. These are amphiphilic compounds produced on living surfaces, mostly microbial cell surfaces, or excreted extracellular and contain hydrophobic and hydrophilic moieties that reduce surface tension and interfacial tensions between individual molecules at the surface and interface, respectively [2]. These are grouped as glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids, polymeric and particulate compounds [7]. Most of these compounds are either anionic or neutral amphiphilic molecules. The hydrophobic part of the molecule is based on long-chain fatty acids, hydroxy fatty acids or α-alkyl-β-hydroxy fatty acids. The hydrophilic portion can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol. When dispersed in water they have the ability to self assemble forming vesicles, micelles, bilayer, microemulsions *etc.* depending on their molecular weight, structure, concentration and experimental conditions. Among these the phospholipids and bile acids have got special attention because of their applicability as delivery systems for drugs, vitamins and cosmetic materials. The biocompatible and biodegradable nature of these makes them a very safe and efficacious vehicle for medical applications [8].

1.1.5 Bile salts as Bio-surfactants:

The salts of bile acids are one of the important biomolecules present in vertebrates including human beings. Bile salts are synthesized from cholesterols in the liver and stored in gallbladder. They act as solubilisers for apolar materials like cholesterol, lipids, and fat-soluble vitamins in the intestine [9]. Therefore they play important role in digestion. The function of bile salts is thus very different from another class of biologically relevant amphiphiles, lipids, which are the building blocks of biological membranes [10]. Apart from these biological applications, bile salts also play an important role as delivery systems for

medicines, cosmetics and several other chemicals because of their unusual solubilizing and emulsifying capacity [11]. The functional characteristics of bile salts are reflected in their specific molecular structure and self assembly behaviour [12].

1.1.6 Structure of Bile Salts:

Bile salts are derivatives of cholanic acid and have a four-ring steroid nucleus and a five-carbon side chain terminating in a carboxylic acid (fig 1.2). The A/B rings are cis and hydrogen on C5 is β and lies below the plane of the molecule. The steroid nucleus of bile acid has the saturated tetracyclic hydrocarbon perhydrocyclopentanophenanthrene, containing three six-member rings (A, B and C) and a five member ring (D). In addition, there is angular methyl groups at positions C-18 and C-19 and a carboxylate group and one to three hydroxyl groups [12]. Bile salts from different species differ chemically in the structure of side chain, stereochemistry of A/B ring fusion and the distribution in number, position and stereochemistry of the hydroxyl groups attached [12]. Depending on the number of hydroxyl groups present bile salts are named as hydrocholate, deoxycholate and cholate in which they have one, two and three hydroxyl groups, respectively.

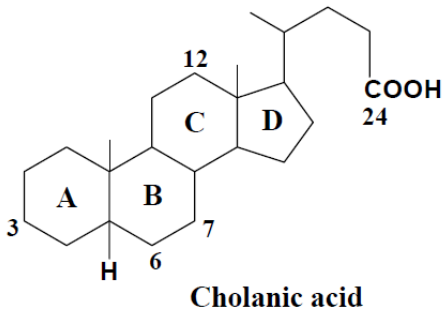
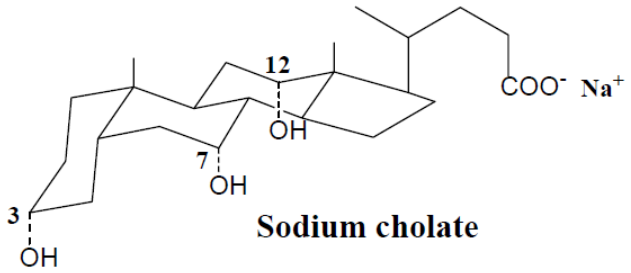
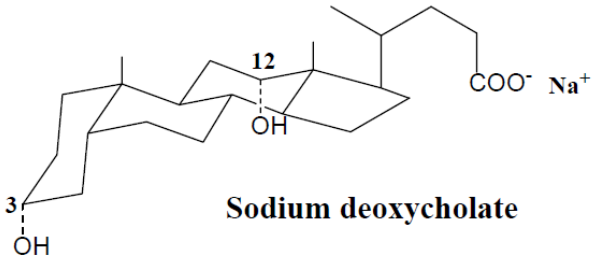


Fig. 1.2 Molecular structure of cholanic acid (5β-cholanic acid)



Sodium cholate



Sodium deoxycholate

Fig. 1.3 Molecular structures of bile salts, sodium cholate and sodium deoxycholate

1.1.7 Amphiphilic Nature of Bile Salts:

The cis nature of A/B rings of the steroidal nucleus gives a somewhat twisted alignment and due to the slightly angled five-carbon chain, the molecule resembles an overturned flattened teaspoon with a short handle (fig. 1.4). The hydroxyl groups lie on the same side of the molecule, on the concave side 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, and is represented by the handle of the spoon. The back of the spoon or the convex side, which includes the methyl groups and the unsubstituted regions of the hydrocarbon rings, forms the hydrophobic domain. The presence of both of these hydrophilic and hydrophobic domains is responsible for the amphiphilic nature of bile salts. Bile salt micelles are somewhat flattened in nature and occupy a relatively large surface area as compared to most of the classical amphiphiles which rather are rod shaped molecules. 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 [13]

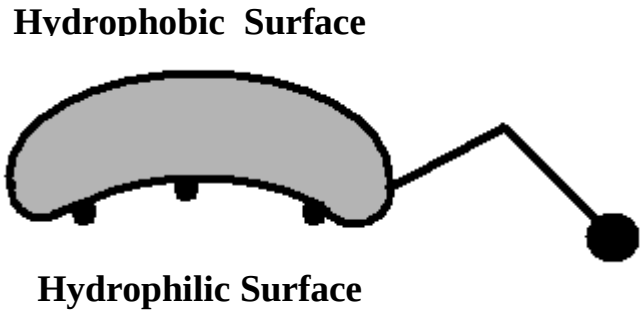


Fig. 1.4 Cartoon representation of sodium cholate showing the amphiphilic nature of a bile salt molecule

1.1.8 Micellisation of Bile Salt

McBain and co-workers in 1940s were first to report the aggregation behaviour of bile salts in aqueous medium and since then extensive study has been carried out by various people on the aggregation behaviour of bile salts [13,14,15,16,17]. Owing to their unique molecular structure, bile salt micelles behave differently from the conventional surfactants. Conventional surfactants show clear-cut polarity gradient between the hydrophilic and hydrophobic parts and both the domains are clearly separated. Whereas bile salts have a facial structure with a convex hydrophobic side and a concave hydrophilic side. The hydrophilic and hydrophobic domains are hence not as clearly separated as in classical amphiphiles [10].

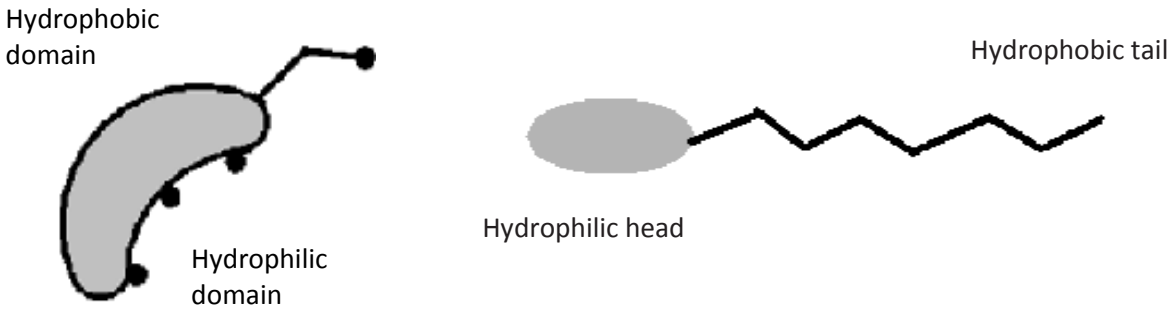


Fig.1.5 Cartoon representation showing difference in structure of bile salt and conventional surfactant.

Bile salts have a smaller aggregation number as compared to the conventional surfactants, for example, two values have been reported for sodium cholate micelles 4 [18] and 16 [18]. It was also suggested that bile salts micelles are smaller in size compared with sodium dodecyl sulfate micelles although their molecular length is almost similar [19].

Micellisation mechanism of bile salts, in fact is still a topic of controversy. The two most debated models in this respect are: i) Small's model of primary and secondary micellisation and ii) Oakenfull and Fisher's model. Out of these two models Small's model is the widely accepted one.

1.1.9 Small's model

D. M. Small's model was based on his study using high-resolution ^{1}H nuclear magnetic resonance (NMR) technique. He has proposed that bile salts form two types of aggregates primary and secondary [13]. Primary micelles consist of two to nine monomers and they are held together by hydrophobic interaction between the steroid nuclei. The primary micelles further aggregate and give rise to large aggregates, held together by hydrogen bonding

between the hydroxyl groups of the primary micelles. The primary micelles are suggested to be globular in shape and the secondary micelles are roughly globular and have oblate ellipsoidal structure.

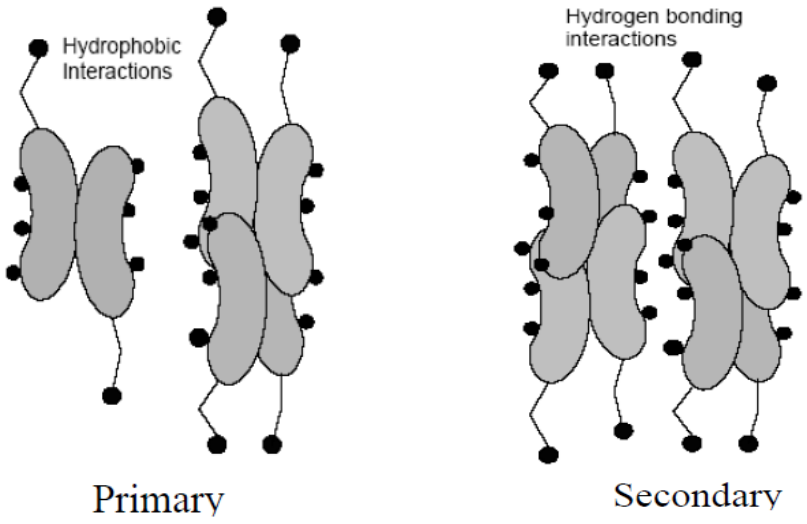


Fig. 1.4 Cartoon representation of bile salt micelles as proposed by Small [13]

1.1.10 Techniques used to study micellisation:

The process of micellisation brings about several changes, which can be related to appreciable alterations in phenomena such as light scattering, surface tension, viscosity and solubilisation of other organic molecules *etc.* These changes are the basis of several analytical techniques that can be employed for the investigation of micelles formation. Thus a number of techniques have been employed for determination of bile salt CMCs, *e.g.* surface tension [13,20,21], viscometry [22,23], solubilisation [24,25,26], light scattering [27], small-angle X-ray scattering [28], neutron scattering [27,29], microcalorimetric titration [30], potentiometry [31,32], electron paramagnetic resonance [33] , nuclear magnetic resonance [34,35,36], spectrophotometry [37,38] and fluorescence technique [39,40,32].

1.2 Application of Bile Salts

The physiological importance of bile salts lies in their ability to solubilise and emulsify cholesterol, dietary lipids and fat-soluble vitamins in the gastrointestinal tract [9]. Apart from their biological importance bile salts also have got much recognition as delivery systems for medicines, cosmetics and several other chemicals [41, 42, 43]. The prime factor that makes bile salts good vehicles for drug delivery is its ability to solubilise hydrophobic dye. Various therapeutic drugs are commercialised as formulations containing bile salts, phospholipids and/or fatty acids. Most often mixed micelles of bile salts and phospholipids along with other components such as cholesterol, other biocompatible surfactants. A commercial product with the trade name Valium MM [43] for *I.V.* application contains the tranquilizer diazepam, a 1,4-benzodiazepin derivative, "solubilised" in mixed micelles (MM). The formulation contains glycocholic acid and soy lecithin as well as benzyl alcohol (preservative), sodium bisulfite, sodium chloride (HCl and NaOH) and water for injection. Progress in the development of highly effective drugs requires the optimisation of existing formulations and the creation of new delivery systems. Keeping this in view detailed interaction studies of several drugs and model drugs with various bile salts are essential. The present work aims at studying the interaction of a model drug a cationic dye Nile Blue A with bile salts (NaDC and NaC).

1.3 Nile Blue A:

Nile blue A (NB) is a cationic benzophenoxazine dye (fig 1.6).

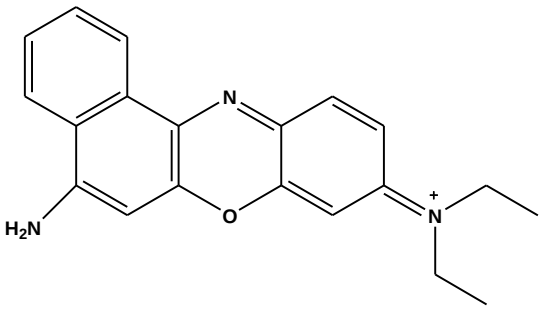


Fig. 1.6 Molecular structure of Nile Blue A

NB has been extensively used as biomarkers and biosensors. It is an excellent DNA binding probe and serves as an intercalator to the stacked base pairs of nucleic acid due to the presence of planar hydrophibic phenoxazine moiety [44]. Ju *et al.* have suggested that NB can be used as an electrochemical indicator for preparation of DNA sensors [45]. It was suggested that NB can be used as a visible dye for DNA detection in gel electrophoresis [46]. NB is an efficient laser dye and has been employed for pulsed and CW operations [47]. NB is a non toxic visible dye having absorption maxima around 638 nm in aqueous medium [48]. The diethylamino group acts as electron donor and iminium group acts as electron acceptors in the molecule.

Although NB is an excellent DNA binding probe and is useful in studying many biological processes related to DNA and proteins, limited reports are available in literature on the interaction of NB with biomimicking self assembled systems.

1.4 Objective:

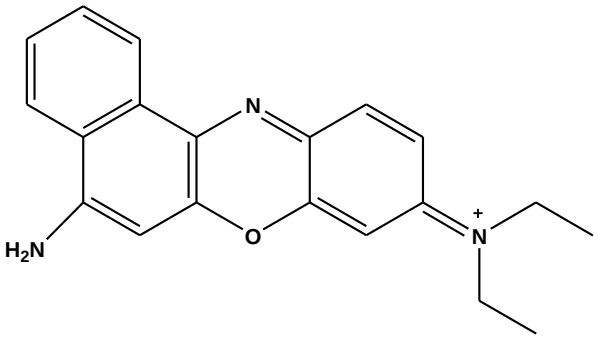
Our aim of the present work is to study the interaction of the cationic dye Nile blue A with bile salts. For this, two bile salts are chosen sodium cholate (NaC) and sodium deoxycholate (NaDC).

MATERIALS AND METHODS

2.1 Materials:

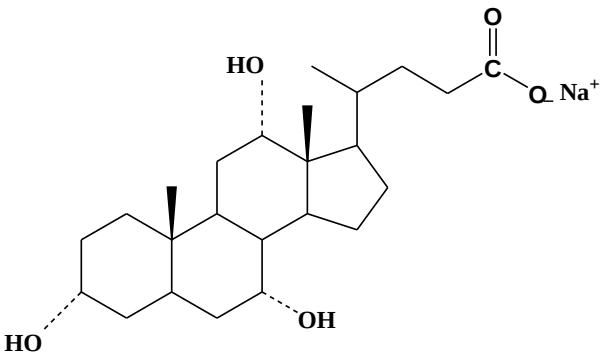
2.1.1 Dye Used

5-amino-9-diethyliminobenzo(a)phenoxazonium sulphate, Nile Blue A (NBA) was purchased from Sigma Co. India and was used without further purification.

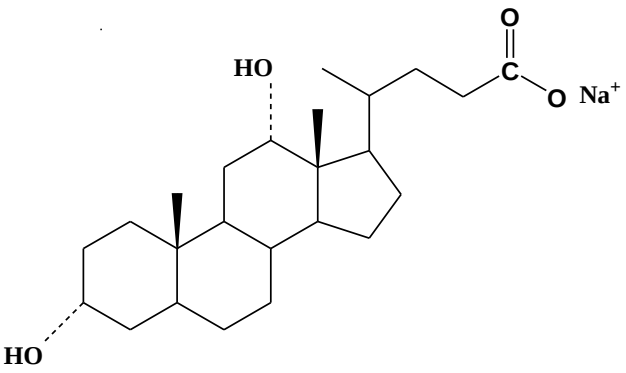


2.1.2 Medium Components

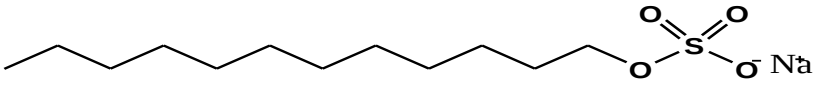
The bile salts, sodium cholate (NaC), sodium deoxycholate (NaDC) were obtained from SRL India. The other surfactants such as CTAB, SDS, Tween 20, were obtained from SD Fine Chemical Co., India. Tx 100 was obtained from Merck India Ltd.



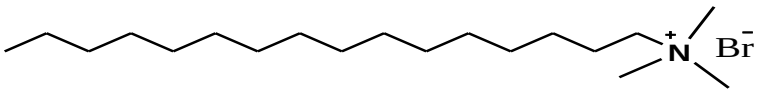
Sodium Cholate (NaC)



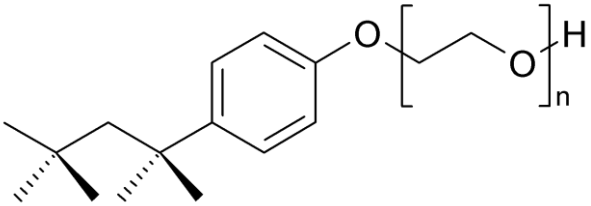
Sodium Deoxycholate (NaDC)



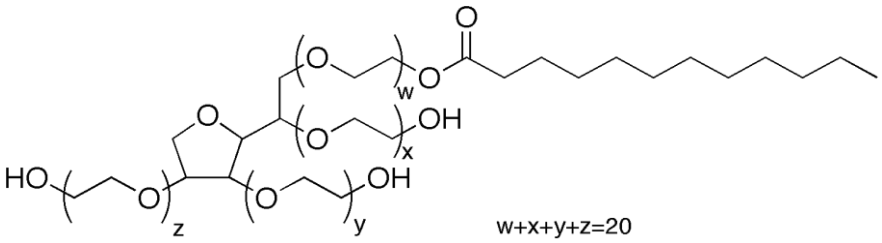
Sodium dodecyl sulfate (SDS)



Cetyl trimethyl ammonium bromide (CTAB)



Triton X 100



Polyoxyethylene sorbitan monolaurate (Tween 20)

2.1.3 Solvents

Deionised water was used for all the experiments. Methanol used was of spectroscopic grade quality and used as received.

2.1.4 Instrumentation

The absorption spectra were recorded using **Shimadzu Spectrophotometer (UV-2450).**

2.2 Methods:

2.2.1 Preparation of Experimental Solutions

The stock solution of NaDC, NaC, CTAB, Tween 20, Tx 100 and SDS were prepared in deionised water and the experimental solutions were made by subsequent dilutions from the

stock solution. Fresh solutions of bile salts were prepared every time. The Nile blue stock solution ($1x10^{-3}$ M) was prepared in methanol. For the experimental solutions the desired amount (μl) of the stock was added to the surfactant solution. The methanol contamination was kept low to avoid any interference with the micellisation process. The concentration of NBA was kept at $1x10^{-5}$ M for the absorption studies.

RESULTS AND DISCUSSION

3.1 Absorption spectrum of Nile blue:

Before going to the interaction study of Nile blue (NB) with bile salt micelles its absorption characteristic in aqueous medium was investigated. Fig 3.1 below represents the spectrum of nile blue in water. As it is clear from the figure the absorption spectrum of NB is broad structureless with a maximum around 631 nm, which matches well with the reported value [49].

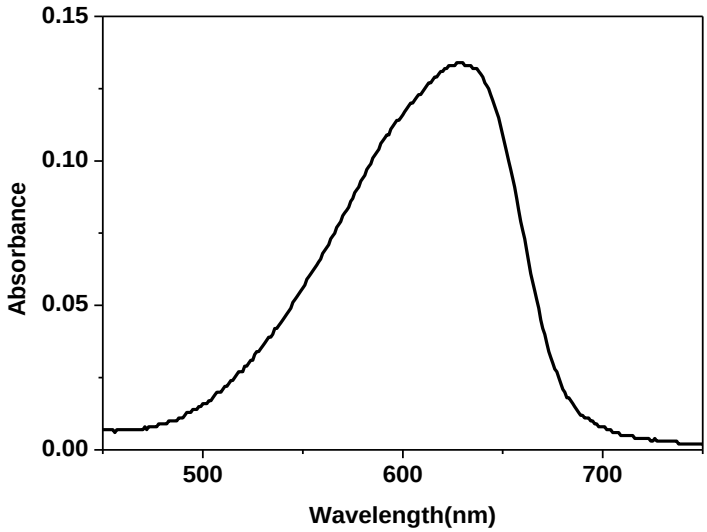


Fig. 3.1 Absorption spectrum of NB in water. Temperature = 25°C, [NB] = 5 x 10^{-6} M

3.2 Absorption spectra of Nile blue in bile salt solutions:

The absorption spectra of NB in NaDC and NaC with increasing concentration of the respective bile salts were studied. Fig. 3.2 represents the absorption spectra of NB with varying concentrations of NaDC.

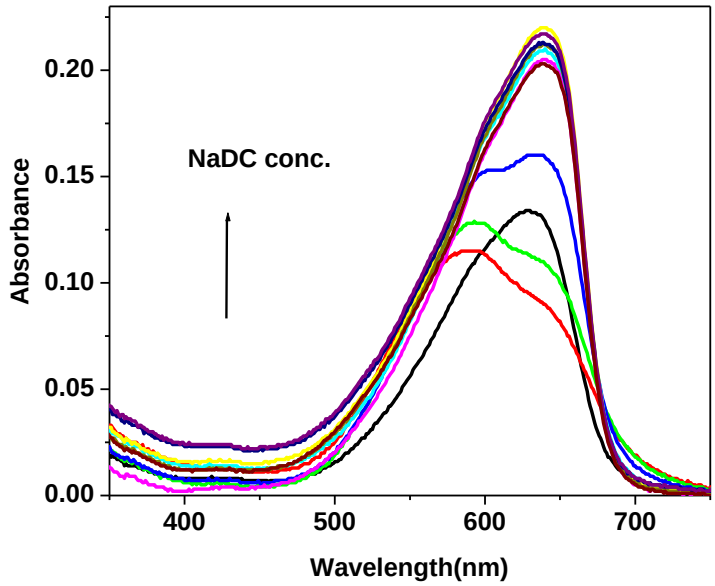


Fig. 3.2 Absorption spectra of NB in water with increasing (A) NaDC concentration. Temperature = 25°C, [NB] = 5 x 10^{-6} M

The absorption spectra of NB in NaDC at higher concentrations of bile salts (above 6 mM for NaDC) was found to be very similar to that of the spectrum of NB in water. The only difference is that in presence of bile salts the absorbance value has increased by almost a factor of two as compared to that in pure water. At lower concentrations of bile salts (below 6 mM for NaDC) the spectra was broadened with a new shoulder peak appearing at the blue end. The absorption spectra of NaC showed similar behaviour. So it can be noted that below the CMC of the respective bile salts NB shows the bifurcated absorption and when the concentration of bile salt is increased above the CMC i.e. when stable micelles are formed NB spectrum looks very similar to that in water but with higher absorbance. This indicates that NB interacts differently with the bile salt monomers and with the micelles. Thus it can be

concluded that bile salt monomers induce aggregation in NB, whereas NB is solubilised in bile salt micelles in monomeric form.

In order to find out the reason for the aggregation of NB in bile salts below the CMC, absorption studies of NB in different charged surfactants like CTAB (cationic surfactant), Tween 20, Triton x 100 (non ionic surfactant) and SDS (anionic surfactant) were carried out.

3.3 Absorption spectra of NB in CTAB:

The absorption spectra of NB with increasing concentration of CTAB are shown in Fig 3.3.

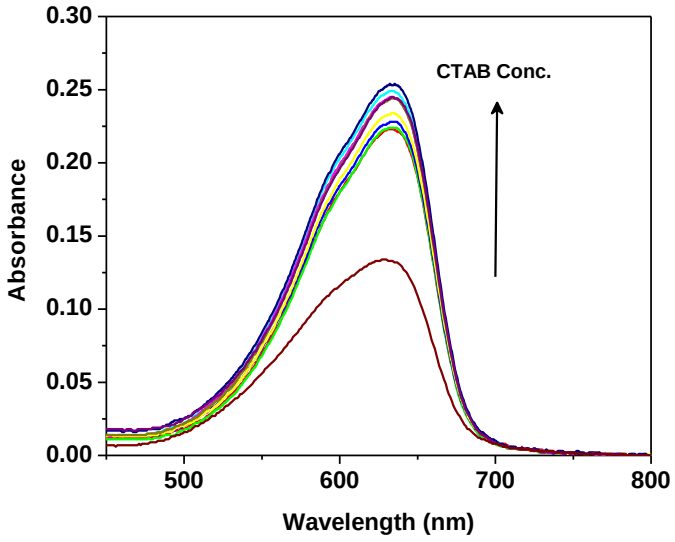


Fig.3.3 Absoprtion spectra of CTAB in water with increasing concentration of CTAB. Temperature = 25°C, [NB] = 5 x 10^{-6} M. [CTAB] = 0-2 mM.

It is clearly seen from the spectra that the spectral profile remains more or less the same in absence and in presence of CTAB. The only difference between the spectrum in water and that in presence of CTAB is that with even a small amount of CTAB the absorbance value of NB increased significantly (almost a two fold increase) and does not show any significant variation with the concentration of CTAB. From this it can be concluded that the interaction of CTAB with NB is similar irrespective of whether the CTAB is in the monomeric form or in the micellar form. There was no sign of the shoulder band in the NB spectra in presence of CTAB.

3.4 Absorption spectra of NB in Tween 20 and in Tx100:

Absorption studies of NB in a non ionic surfactant Tween 20 and Tx100 were carried out. The spectral profile of NB remained almost similar to that in water and shoulder band was not seen. However, the absorbance at the maximum kept on increasing with the increasing concentration of both the surfactants.

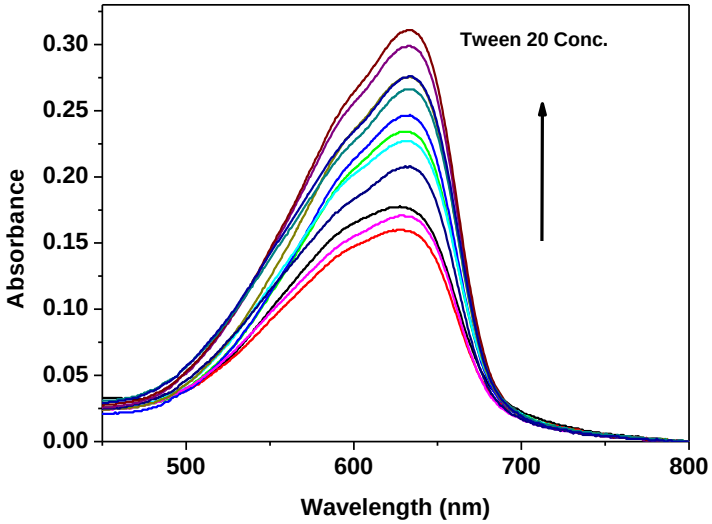


Fig. 3.4 Absorption spectra of NB in water with increasing concentration of Tween 20 Temperature = 25°C, [NB] = 5 x 10^{-6} M. [Tween 20] = 0-0.5 mM.

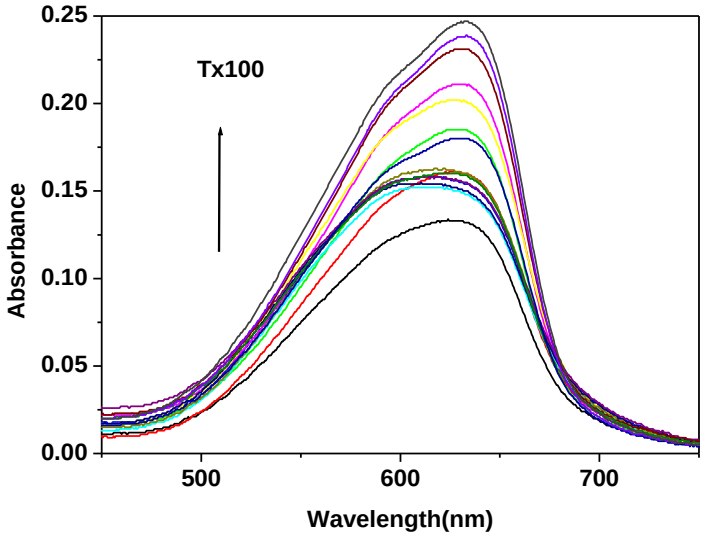


Fig. 3.5 Absorption spectra of NB in water with increasing concentration of Tx100 Temperature = 25°C, [NB] = 5 x 10^{-6} M. [Tx100] = 0-0.34 mM.

3.5 Absorption spectra of NB in SDS:

The absorption spectra of NB with increasing concentration of the anionic surfactant SDS were recorded. The spectral behaviour of NB in SDS matched closely with that of the spectra of NB in bile salts. Therefore it can be concluded that the anionic surfactant SDS also induces aggregation in NB below the CMC and above the CMC the NB monomers get solubilised in the micelle core.

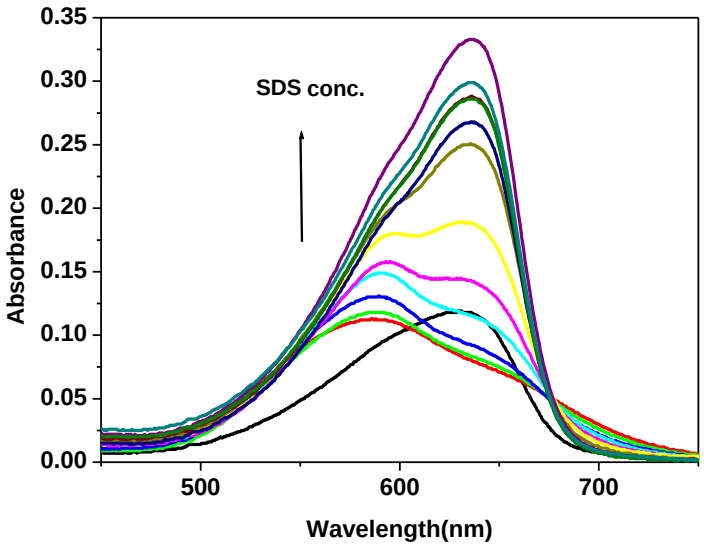


Fig. 3.6 Absorption spectra of NB in water with increasing concentration of SDS Temperature = 25°C, [NB] = 5 x 10^{-6} M. [SDS] = 0-20 mM.

From the above study it is clear that the interaction of NB with a surfactant depends on the nature of the surfactant. Out of all the surfactants studied the spectral profile of NB was found to be similar in CTAB, Tween 20 and Tx 100, which differs from that in bile salts and SDS. It is already discussed in the introduction chapter that conventional surfactants are structurally very different from that of bile salts. But the spectral behaviour of NB was found to be similar in SDS and bile salts, and SDS and bile salts have only one thing in common that is the charge both being negatively charged. So it can be concluded that the aggregation of the cationic dye NB in SDS and bile salts below their respective CMCs is charge induced.

Conclusions

- Bile salts below the CMC induce aggregation in the cationic dye nile blue.
- Above the CMC the NB monomers get solubilised in hydrophobic micellar core.
- Nile blue shows similar spectral behavior in SDS as that in bile salts.
- NB shows different behaviour with CTAB (cationic surfactant), Tween 20 and Tx100 (non ionic surfactants).
- The aggregation process is charge induced. The aggregation of NB is due to the charge neutralisation by negatively charged surfactant monomer followed by hydrophobic interaction between the dye molecules.

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