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Fluorescence Spectroscopic Analysis of Sodium Cholate Induced Disaggregation of Methylene Blue

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Abstract: Methylene blue (MB) has been used as a staining reagent for a long time. And also it has been established as a pharmaceutical agent recently. MB in aqueous environment undergoes self-association and forms dimmers, followed by formation of larger aggregates. The absorption studies reveal presence of monomer and dimer at 665 nm and 610 nm respectively, while the higher aggregates were found at around 292 nm. MB is a well-known staining dye and also a fluorescent molecule. Even though MB has a strong fluorescence, the adaptation of MB as a fluorescent probe is not explored thoroughly. Here, we attempt to probe the micellization behavior of bile salt media by using MB monomer absorption and fluorescence. Bile salts are well known disaggregation agents and widely used as drug delivery systems. Among the various bile salts, sodium cholate (NaC), a primary unconjugated bile salt was chosen for the study. The absorption and fluorescence data indicate that there is increase in absorbance and fluorescence intensity along with increase in fluorescence anisotropy. The study reveals that NaC induces disaggregation of MB aggregates through hydrophobic interactions.
Keywords: Methylene blue, Sodium cholate, Bile salts, Absorption, Fluorescence spectroscopy, Aggregates.

Introduction

Methylene blue (MB) has been used as a staining reagent for a long time and also it has been established as a pharmaceutical agent recently.[1,2] MB has recently been reported to be effective arresting the progress of Alzheimer's and other neurodegenerative disease.[1,2] The absorption spectrum of MB has been widely studied in order to fully understand its chemical behavior.[3] MB exhibits two major absorption bands at 293 nm (π-π^* transition) and 665 nm (n-π^* transition) in dilute aqueous solutions, with a shoulder at 610 nm correspondingly to the 0-1 vibronic transition.[4] MB undergoes spontaneous self-aggregation under physiological conditions. Many spectral studies show that the monomer absorption peak is at 665 nm, while the dimer peak is at 610 nm and the larger aggregates around 293 nm.[5,6] Spectral properties of MB has been studying in both homogenous and organized media like proteins, surfactants etc.[4-10] Recently MB has been used as a pharmaceutical agent and its formulation is quite challenging due to its self-association in aqueous media. Hence it is important to disaggregate the MB aggregates prior to their formulation. Conversely, if a disaggregating agent can be used as a drug delivery system or a pharmaceutical excipient to avoid the aggregation of MB.

Bile salts are biological compounds that are synthesized from cholesterol in the liver. They are typically composed of a steroidal backbone with one or more alpha oriented hydroxyl groups unconjugated to an anionic chain or tail.[11] Bile salts are widely used as solubilization and disaggregating agents in the pharmaceutical industry.[12,13] In aqueous media, bile salts undergo spontaneous aggregation leading to primary and secondary aggregates involving a step – wise aggregation model.[14] The primary aggregates are formed due to hydrophobic interaction between the steroidal back-bone. Secondary micelles are held together by hydrogen bond. Among the various bile salts, sodium cholate (NaC), a primary unconjugated bile salt was chosen for the study.

Materials and Methods

Methylene blue and Sodium cholate were purchased from Sisco Research Laboratories Private Limited (India) and were used without any further purification. A sodium phosphate buffer of pH 7.4 with 50 mM concentration was used for all experiments. The solvent ethanol used was of spectroscopic grade. The stock solutions of MB are prepared by dissolving it in ethanol and the required concentration of MB solution was prepared by further dilution with pH buffer. UV-visible spectrophotometric experiments were recorded on a Shimadzu UV 1800 double beam spectrophotometer, using 10 mm quartz cuvettes. A Horiba Jobin-Yvon Fluoromax-4P spectrofluorometer was used to record the fluorescence spectra. Excitation and emission spectra were measured with 5 nm band width. Temperature related experiments were done in a double walled cuvette holder carrying the fluorimetric cell. The temperature was controlled by circulating water through jacketed cuvette holder from a refrigerated bath (JULABO, Germany). The steady state fluorescence anisotropy is defined as, [15]

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

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

$$G = I_{HV} / I_{HH}$$

Preparation of MB - NaC solutions

The stock solutions of NaC were prepared in 50 mM phosphate buffer pH 7.4 at concentration higher, than the critical micellar concentration (cmc) (NaC = 6-16 mM). A range of different concentrations of bile salt solutions are prepared by diluting these stock solutions using buffer solution. MB concentration was maintained at 5 µM for all the experiments with less than 2% ethanol contamination. The range of concentration of bile salt varied is NaC = 4.8 – 43.2 mM. The solutions were incubated for 2 hrs to achieve equilibrium.

Result and Discussion

Absorption Spectra of MB in presence of NaC

The absorption spectrum of MB is given in (Fig.1). It exhibits a peak at 665 nm corresponding to the MB monomer, a shoulder at 610 nm corresponding to MB dimer and higher aggregates of MB at around 292 nm.[4-9] The MB absorption increases with the addition of NaC.

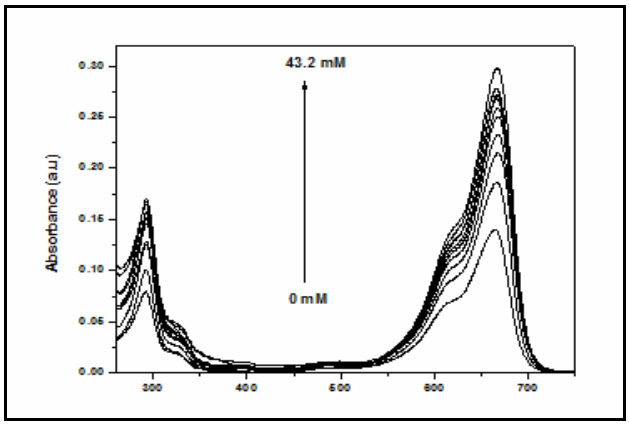

Figure 1. Effect of NaC on absorption spectra of MB, T = 25 °C and pH = 7.4

The concentration range of NaC solution used here reflects the critical micellar concentration (cmc) range of the NaC aggregates, *viz.* 6 – 16 mM.[12-14] The lower value in the cmc range indicates the initial formation of the primary (dimer) aggregates of NaC, while the higher value in cmc range indicates the formation of secondary (larger) aggregates. The enhancement of MB monomer absorbance at 665 nm, reveals a possible solubilization of MB aggregates by NaC media and thereby disaggregation of MB aggregates.

Fluorescence spectra of MB in presence of NaC

The fluorescence spectra of MB when excited at 665 nm shows emission peak at 686 nm (Fig 2) corresponding to the monomer fluorescence. The results indicate that there is an enhancement of fluorescence intensity of MB monomer. There is a small shift in the emission wavelength to the red region. The fluorescence anisotropy was measured at 686 nm emission peak.

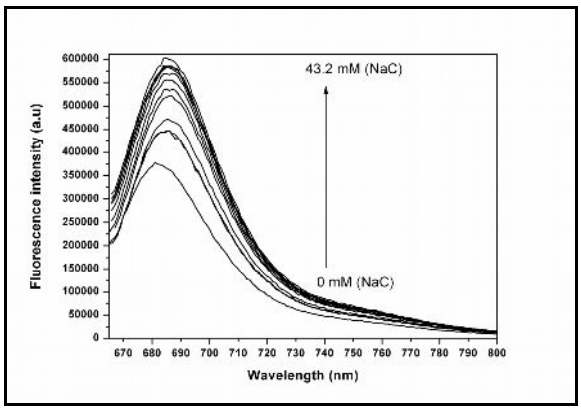

Figure 2. Effect of NaC on fluorescence emission spectra of MB (λ_{ex} 665 nm, λ_{em} 686 nm), T = 25 °C and pH = 7.4

The enhancement of fluorescence intensity of MB with the increasing concentration of NaC solution is given by figure 3. It indicates that the increase in fluorescence intensity follows the micellization of NaC aggregates along with the disaggregation of MB aggregates.

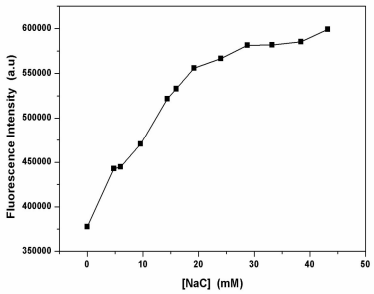

Figure 3. Plot of fluorescence intensity of MB (λ_{ex} 665 nm, λ_{em} 686 nm) with the addition of NaC, T = 25 °C and pH = 7.4

Steady state fluorescence anisotropy of MB

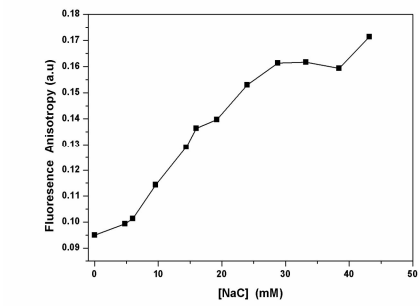

Figure 4. Plot of fluorescence anisotropy of MB (λ_{ex} 665 nm, λ_{em} 686 nm) with the addition of NaC, T = 25 °C and pH = 7.4

The fluorescence anisotropy (r_{ss}) of MB monomer with the addition of NaC was measured at 686 nm (λ_{ex} 665 nm) at 25 ^{0}C (Fig. 4). The r_{ss} values of MB monomer increases from 0.10 to 0.18 with the increasing concentrations of NaC aggregates. Even though the change in fluorescence anisotropy is minimal, the r_{ss} values

in collaboration with the enhancement in fluorescence intensity of MB with increasing concentration of NaC solutions clearly indicate that the MB monomer associates with the hydrophobic core of the NaC aggregates.

Disaggregation of MB by NaC media

The photophysical parameters of MB monomer, namely absorbance (λ_{ex} 665 nm), fluorescence intensity (λ_{ex} 665 nm, λ_{em} 686 nm) and fluorescence anisotropy (λ_{ex} 665 nm, λ_{em} 686 nm) that the NaC media used here is effective in solubilizing the MB monomer and thereby disaggregation of MB aggregates is observed. The results indicate that the interaction between MB and NaC is essentially hydrophobic in nature. The rod like structure representing the hydrophobic region of MB interacts with the steroidal domain of the NaC molecules. Hence MB monomer can easily associate with the dimer aggregate of NaC and gets incorporated within the hydrophobic core of the larger secondary aggregates of NaC. Thereby, the disaggregation of MB aggregates induced by NaC media and MB monomers are abstracted within the NaC aggregates.

Conclusion

The absorption and fluorescence studies of MB molecules show that MB exists as monomer, dimer and higher aggregates. MB monomer absorption is at 665 nm and emission is at 686 nm. The increase in absorbance and fluorescence intensity of the monomer MB with increasing bile salt concentrations indicate the monomerization of MB aggregates induced by NaC media. This is further proved by steady state fluorescence anisotropy studies. The photophysical parameters such as absorbance, fluorescence intensity and fluorescence anisotropy indicate that the nature of interaction between MB and NaC media are essentially hydrophobic. Thus, NaC molecules can be used as disaggregating agents/ drug delivery media in the formulation of MB.

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