



Thiazine dyes: Evaluation of monomeric and aggregate forms



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ABSTRACT

The aggregation phenomenon of Azure B, monobrominated Azure B, Thionine and Methylene Blue was studied by UV–Visible spectrophotometry in different media as a function of dye concentration and temperature variations. The tests carried out in organic solvents allowed the identification of monomeric species of these compounds, which have not been reported in literature and have been wrongly assigned for years. The results obtained in water allowed demonstrating that different kinds of aggregates are present in this medium. In addition, the aggregation tendency of these dyes in organic solvent and aqueous media was established. Several parameters such as lipophilicity, effect of bulky substituents and interactions with media were considered to interpret the aggregation behavior of thiazine dyes.

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1. Introduction

The aggregation phenomenon involves the association between solute molecules in solution and gives assemblies (aggregates) of individual units (monomers). The phenomenon arises when intermolecular interactions between solute molecules become energetically more favorable than the interaction between solute and solvent molecules. The aggregation may involve interaction between two or more solutes (hetero-aggregation), but most examples that have been studied involve self-aggregation [1].

The molecules are held together by weak interaction forces, which may include hydrogen bonding, electrostatic interaction including π – π interactions, Van der Waals and hydrophobic interactions, depending on the characteristics of the structure [1,2]. Molecular exciton theory of dipole–dipole coupling is one of the well accepted theoretical tools by which different types of dye aggregates (H, J or intermediate) could conveniently be analyzed. According to this model, parallel aggregates (H aggregates) exhibit an absorption band at shorter wavelengths than their monomers, while head-to-tail aggregates (J aggregates) show a red-shift band [3,4].

The molecular association is strongly affected by several parameters such as structure and concentration of solute, temperature, ionic strength and presence of organic solvents. The aggregation may increase with an increase in dye concentration or ionic strength or decrease with the rise in temperature or the addition of organic solvents. Besides, solubilizing groups (as sulfonate group) in the dye structure

decrease aggregation, whereas the inclusion of alkyl chains increases aggregation because of the higher hydrophobic interaction in solution [4,5]. However, it has been demonstrated that *t*-butyl group increases the monomer concentration in solution because this bulky substituent produces a steric hindrance between the dye molecules [6].

The molecular interaction leading to aggregation is one of the most important features of organic dyes in solution since it affects their color, solubility and photophysical properties. Therefore, it is important to evaluate the aggregation of these compounds in solution [4]. It is well known that the ionic dyes tend to aggregate in diluted solutions, leading to dimer formation, and sometimes to even higher order aggregates. Interactions among dye molecules produce significant spectral shifts and distinct changes in band shape. For this reason, it was suggested that to explain the substantial deviations from Beer's law observed for highly colored organic ions such as Methylene Blue (MB), Thionine (Th), Crystal Violet and certain cyanine and xanthene dyes, the aggregation of dyes should be considered [7]. The self-association of these compounds has fundamental consequences for applications as diverse as photographic technology, tunable lasers, molecular optoelectronic devices, fluorescence depolarization diagnostic devices, and photomedicine [8]. In photodynamic therapy (PDT) and photodynamic antimicrobial chemotherapy (PACT), an adequate concentration of dye (photosensitizer) is required at the target site for a sufficient time in order to produce damage at cellular level. The formation of aggregates could significantly alter the biologic activity of photosensitizers since only monomeric species are appreciably photoactive [9].

Thiazines are a class of electron-rich tricyclic nitrogen–sulfur heterocycles with a low oxidation potential and a high propensity to form stable radical cations. Although these dyes have a planar aromatic molecular structure with an intrinsic cationic charge, they exhibit

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relatively intense luminescence, high photoconductivity and undergo reversible oxidation processes, and for this reason, they are widely used as dyes, antioxidants, and staining for microscopy and pharmaceuticals [10–12].

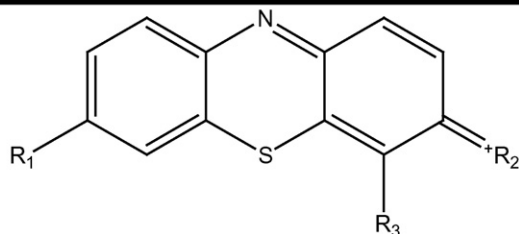
There are numerous aggregation studies on thiazine dyes, mainly about Th and MB, but surprisingly their results are frequently inconsistent and sometimes contradictory. For example, the value of dimeric constant of MB varies from $4 \times 10^{-3} \text{ M}^{-1}$ to $6 \times 10^3 \text{ M}^{-1}$ in aqueous solutions at 25–30 °C [4,13,14]. This unsatisfactory situation arises from experimental conditions wrongly defined and numerous assumptions made in the processing of the spectral data [5]. All of them assume that the monomeric form is present in aqueous solutions even at high concentration of dye, although there are no experimental data on aggregation of thiazine dyes in monomerizing solvents at very low concentration of dye [13–15]. In addition, several publications suggest a possible structure for dimers of thiazine dyes but it has not been characterized yet [16–18].

Hence, the objective of the present study was to evaluate the aggregation effect of Azure B (AzB) and its novel monobrominated derivative (AzBBr), belonging to thiazine dyes, in different media as a function of dye concentration and temperature by UV–Visible spectrophotometry, a methodology widely used to evaluate this phenomenon. In order to analyze the aggregation tendency of this family of dyes, the aggregation of Th and MB was evaluated because these dyes are the most studied thiazines. Additionally, the values of the dimerization constants of these dyes were calculated by DECOM. All studied compounds bear the same dye skeleton, but differ in the substituent groups (Scheme 1).

2. Experimental

2.1. Materials and methods

The commercial phenothiazine dyes investigated (Th, MB and AzB) were purchased from Sigma Aldrich Co. (St. Louis, MO) and studied without additional purification. The monobrominated derivative (AzBBr) was obtained by the method previously described [19]. The purity of these dyes was corroborated by reversed-phase high-performance liquid chromatography (RP-HPLC) using an Agilent 1100 Series HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler tray, a thermostatted column compartment, a UV–Vis detector and a reversed-phase C₁₈ column (Supelco®, 4.6 mm × 250 mm, 5 μm) with guard column and a flow rate of 1.0 mL/min. Instrumental control and chromatographic data acquisition were performed by the Agilent ChemStation (Rev. B.03.01) software.



Compounds	R ₁	R ₂	R ₃
AzB	N(CH ₃) ₂	NH(CH ₃)	H
AzBBr	N(CH ₃) ₂	NH(CH ₃)	Br
Th	NH ₂	NH ₂	H
MB	N(CH ₃) ₂	N(CH ₃) ₂	H

Scheme 1. Structure of thiazine dyes.

The mobile-phase was prepared with methanol HPLC grade and an aqueous solution of trimethylammonium phosphate 83 mM (70:30 v/v). All dyes were used at purity levels between 92% and 98%.

The solvents used for spectrophotometric measurements were of pro-analysis grade (Cicarelli, Sintorgan, Anedra) and the aqueous solutions were prepared using ultrapure water from the Milli-Q® water purification system (Millipore Corporation, USA).

Absorption spectra in the range 350–750 nm were measured with an Evolution 300 spectrophotometer (Thermo Scientific, Madison, USA), using a 1 cm path length quartz cell. To corroborate the results obtained at low dye concentrations, some measurements were performed in a Shimadzu UV-1800 spectrophotometer using a 5 cm path length cell. The results obtained by spectrophotometric measurements were processed with OriginPro 8 SR0 (OriginLab Corporation).

2.2. Aggregation behavior as a function of dyes concentration

The stock solutions of dyes (0.1 mg/mL) were prepared in different media and then diluted appropriately with the same solvent to obtain solutions from 10^{-7} M to 10^{-5} M . AzB and its derivative AzBBr were evaluated in *N,N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO), ethanol, mixtures of ethanol: water and pure water. In addition, the aggregation behavior of Th and MB was studied in DMF and pure water to evaluate the monomer-dimer balance and formation of higher order aggregates, respectively. All experiments were carried out at least twice with consistent results.

2.3. Aggregation behavior as a function of temperature

Aggregation behavior of AzB, AzBBr, Th and MB as a function of temperature was evaluated by UV–Visible Spectroscopy. The stock solutions of each dye (0.1 mg/mL) were prepared in DMF and then diluted appropriately with the same solvent to obtain three solutions of between 10^{-6} M and 10^{-5} M . Aliquots of each solution (2 mL) were placed in a thermostatic bath (Haake F3–K), at temperatures from 25 °C to 75 °C before analyzing.

2.4. Determination of dimerization constant

The dimerization constant (K_d) of thiazine dyes was calculated from experimental curves in DMF by DECOM program. This Add-in implementation provides a conventional, reliable, and easy to use tool for the study of dimer formation equilibrium using electronic absorption spectroscopy [20].

The linear and nonlinear least-squares fitting methods were used for the calculation of $\log K_d$ values. These methods consist of two main parts including determination of the dimerization constant and spectral decomposition. A detailed description of these methods and their implementation as an Add-in, have recently been reported [20,21].

3. Results and discussion

3.1. Aggregation behavior as a function of dyes concentration

3.1.1. Aggregation behavior of AzBBr and AzB in pure organic solvents

The UV–Visible spectra obtained for AzBBr dye in DMF in the range of concentrations studied were reproduced in Fig. 1A. A unique band was observed for this dye at low concentrations (<9.5 μM) with a maximum absorption at 534 nm. The increase in dye concentration caused both an increase in the intensity of this band and the appearance of a new band at 655 nm.

It is well known that DMF is a monomerizing solvent widely used in aggregation studies to evaluate the monomeric species [22,23]. For this reason, the monomer of AzBBr was assigned to the hypsochromic band (534 nm), while the band that appeared at higher wavelength (655 nm) corresponds to J-aggregate. This aggregate might be related to the

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