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# Thiazine dyes: Evaluation of monomeric and aggregate forms

Jimena Vara, Cristina S. Ortiz *

*Departamento de Farmacia, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, X5000HUA Córdoba, Argentina*

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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.

## 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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* Corresponding author at: Facultad de Ciencias Químicas, Ciudad Universitaria, Universidad Nacional de Córdoba, Córdoba, Argentina.
*E-mail address:* crisar@fcq.unc.edu.ar (C.S. Ortiz).

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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].

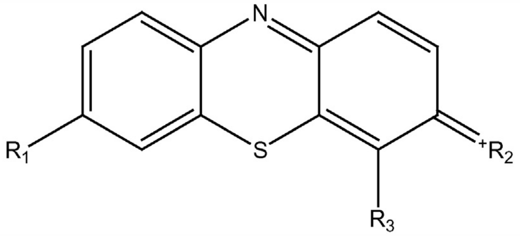
There are numerous aggregation studies on thiazine dyes, mainly about Th and MB, but surprisingly their results are frequently inconsistent and sometimes contradictory. For example, the value of dimeric constant of MB varies from $4 \times 10^{-3}$ $M^{-1}$ to $6 \times 10^{3}$ $M^{-1}$ in aqueous solutions 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 $C_{18}$ 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. 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%.



| 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 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 (<t;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

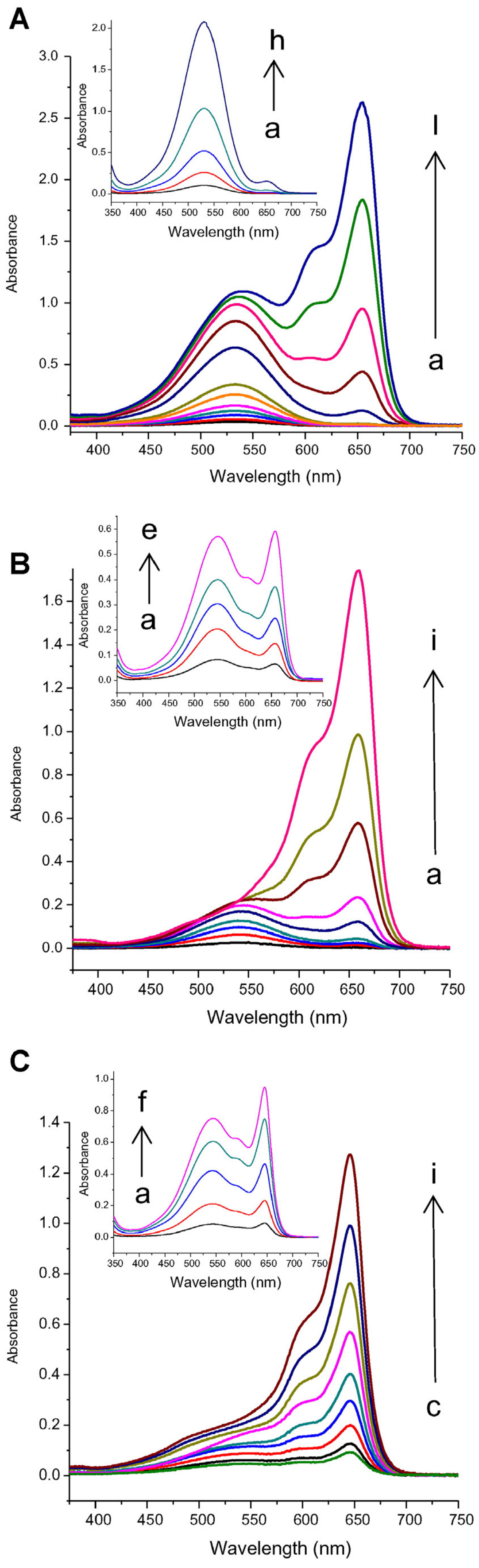


**Fig. 1.** Absorption spectra recorded at different concentrations of AzBBr in (A) DMF, (B) DMSO and (C) ethanol. Inset: Absorption spectra using a 5 cm path length quartz cell. [AzBBr] (a) 0.9 μM, (c) 3.5 μM, (e) 7.1 μM, (f) 9.5 μM, (h) 18.9 μM, (i) 28.4 μM and (l) 56.8 μM.

dimeric structure of the evaluated compound. Additionally, the Beer-Lambert law was not obeyed at monomer (S1A-Supporting information) or aggregate (S1B-Supporting information) maximum absorption wavelengths, which was related to the effects of dye aggregation.

These results were comparable to those obtained for AzB dye in the same solvent with the particularity that in the range of concentrations studied, the spectra presented two absorption bands (Fig. 2A): one of them which has not been described in literature yet, and to which we attributed the monomeric form, at 520 nm, and a second one with a maximum absorption wavelength at 649 nm, which evidenced the formation of J-aggregate. The Beer-Lambert law was not obeyed at any of the maximum absorption wavelengths, corresponding to the monomeric (520 nm) and aggregate forms (649 nm) (data not shown).

The aggregation phenomenon of both dyes, AzBBr and AzB, was also evaluated in DMSO, which is another monomerizing solvent extensively used [22,23]. The brominated derivative showed a clear balance between the monomeric and J-aggregate forms in the range of concentrations studied (Fig. 1B). The presumed monomer presented a band at 540 nm while the maximum absorption at 659 nm was attributed to the dimeric form, which became the predominant species at higher concentrations than 7.1 μM. The absorption spectra of AzBBr in DMF and DMSO shown in Fig. 1A and B, respectively, demonstrated that the absorption characteristics were similar in both solvents but in DMF; the monomeric form existed as the unique species in diluted solutions, and the monomer-dimer equilibrium was observed at a higher concentration of dye than that detected in DMSO.

In the case of AzB in DMSO (Fig. 2B), a unique aggregate band with a maximum absorption at 654 nm was detected. Important evidence was observed in normalized spectral curves at maximum absorption wavelength (Fig. 2B, inset). It was noted that the absorbance between 475 nm and 575 nm decreased when the dye concentration increased. This effect could be attributed exclusively to the monomeric species of AzB dye.

In ethanol, both dyes showed a predominant absorbance maximum, corresponding to the J-aggregate. The monobrominated derivative presented an absorption maximum at 648 nm (Fig. 1C), while the normalized absorption spectra at this wavelength of this dye showed that the absorbance around 550 nm decreased when the dye concentration increased (S2-Supporting information); this effect could be attributed to the presence of the monomeric species. To corroborate these results, the measurements were performed using a 5 cm path length cell (Fig. 1C, inset). The obtained curves allowed us to clearly see a band at 545 nm and a maximum at 648 nm. Thus, we proved the presence of monomer at low concentrations of AzBBr with a maximum of around 550 nm, as well as assigned the bathochromic band to the formation of J-aggregates. The spectral curves of AzB dye obtained in ethanol were overlapped in all concentration ranges studied and presented an absorption maximum at 640 nm, ascribed to the presumed dimer. In this case, the absorption band of the monomeric species was not observed (data not shown).

These studies allowed identifying the correct monomer species of thiazine dyes. The balance between monomer and J-aggregate was observed for both dyes, AzBBr and AzB, in most of the solvents studied. These results confirm that the organic solvents favor the disruption of the aggregates. In addition, under our experimental conditions, the monomeric species of AzBBr was more favored than the monomer of the precursor (AzB). The monomeric form of the brominated derivative was the only species detected in DMF at low concentrations of dye (<9.5 μM, Fig. 1A) and was also the predominant form at higher concentrations than that of AzB dye (Fig. 2A). Additionally, the monomer of AzBBr dye was observed in a less monomerizing solvent, such as ethanol (Fig. 1C, inset). This effect could be attributed to the steric hindrance caused by the incorporation of a bulky substituent, such as bromine, which hinders the approach of the dye molecules, and thus the intermolecular interactions that stabilize the aggregate [24]. Several studies show that the dimerization constant of a series of compounds decreases

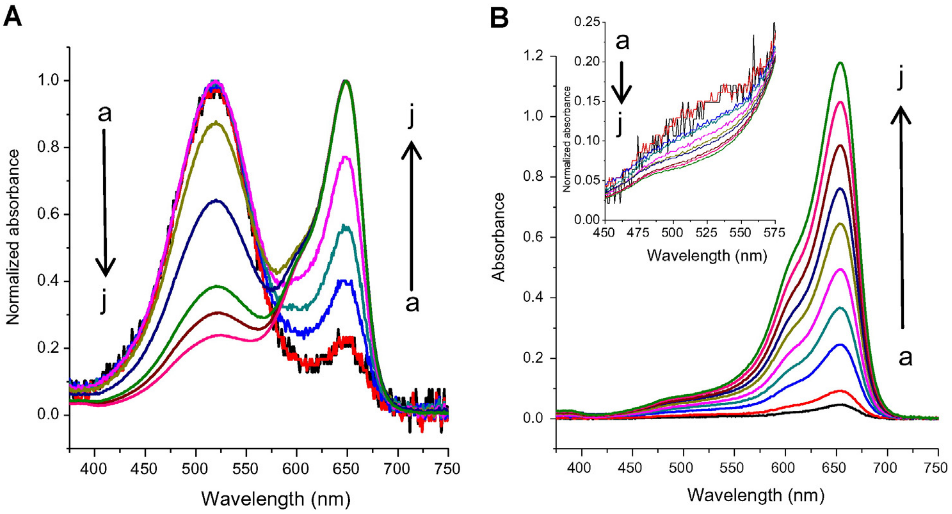


**Fig. 2.** A- Normalized absorption spectra of AzB at maximum absorption wavelength in DMF as a function of dye concentration. [AzB] (a) 1.3 μM and (j) 49.1 μM. B- Absorption spectra of AzB in DMSO recorded at different concentrations. [AzB] (a) 0.7 μM and (j) 14.7 μM. Inset: Normalized absorption spectra at maximum absorption wavelength as a function of dye concentration from 450 nm to 575 nm.

when the volume of substituents increases. Monahan et al. proved that replacing a methyl group with an ethyl or isopropyl substituent produces significant changes in the stability of the dimeric form, which is reflected in the decrease of dimerization constants [25]. Other authors have demonstrated that bulky substituents as *tert*-butylsulfonyl group, present higher inhibition of aggregation than long alkyl chains [6,22]. However, less bulky groups such as methane have reported significant steric effects on the aggregation of dyes, producing a marked decrease in dimerization constant [4]. Note that the van der Waals radius of the bromine atom and the methyl group is similar; therefore, the steric hindrance caused by them can be comparable [26,27]. In addition, previous studies have shown that the bromination of azine dyes, compounds with similar structure to that of the thiazines, causes a decrease in the formation of aggregation [28].

In this work, the effect caused by the increase in the concentration of dyes was analyzed as a parameter directly related to the formation of aggregates. This effect was then compared in different solvents. The aggregation phenomenon should not be confused with solvatochromism which is used to analyze the spectral shifts caused by media variations when evaluating a single concentration of the compound [29]. The monomer and J-aggregate species of AzBBr and AzB dyes were analyzed at the same concentration of dye in DMF, DMSO and ethanol in order to evaluate the solvatochromic effect. A positive solvatochromism was observed for both species of dyes. This behavior corresponds to the bathochromic shifts of the UV/Vis absorption band when the solvent polarity increases [30]. The most commonly used parameters to determine the polarity of the solvents are the dielectric constant and the $E_T(30)$ scale [31,32]. The former parameter accounts for the effect of the environment on the weakening of the interaction between charges, while the latter is a descriptor of both hydrogen bond and electrostatic interactions of solvents.

A bathochromic shift of the monomeric species of AzBBr was observed as the $E_T(30)$ values of the solvents increased ($E_T(30)$ values: 43.8(DMF); 45 (DMSO); 51.9 (ethanol) - S3A-Supporting information). Moreover, the J-aggregate of this dye showed a band at 648 nm, 655 nm and 659 nm in ethanol, DMF and DMSO, respectively (S3B-Supporting information). This behavior is related to a positive solvatochromic effect if we consider the dielectric constant as an indicator of the polarity of the solvent. The obtained results evidence that the formation of dimers involves different intramolecular interactions which affect the bonds between the dye and the solvent. The monomer species may generate electrostatic interactions and hydrogen bonds with the environment, while the solvation of dimers would be more influenced by electrostatic interactions.

### 3.1.2. Aggregation behavior of AzBBr and AzB in ethanol: water mixture and pure water

The normalized spectral curves of AzBBr (Fig. 3) and AzB (data not shown) in ethanol:water (75:25 v/v) showed a complete overlap in the range of concentrations evaluated, with a maximum absorption wavelength at 649 nm and 644 nm, respectively. Note that these bands present a hypochromic shoulder (≈600 nm) attributed to vibronic transitions from the lowest vibrational sublevel in the ground state to the second sublevel in the excited state, which is independent of dye concentration [33].These spectra agree with those attributed to J-aggregate of each dye in pure organic solvents (Section 3.1.1). Under these experimental conditions, the monomeric bands of both compounds were not detected. Fig. 3A shows the normalized curves of AzBBr dye in ethanol:water (75:25 v/v). As expected, in this medium AzBBr and AzB dyes obeyed the Beer-Lambert in concentration ranges, indicating the existence of a unique species in solution (data not shown).

An increase in the absorbance of the hypsochromic shoulder was observed in ethanol:water (10:90 v/v) when the concentration of AzBBr (Fig. 3B) and AzB (data not shown) dyes was increased. This shoulder for the monobrominated derivative became a new absorption band with a maximum absorption at 607 nm.

In pure water, the shoulder for both dyes increased significantly. These results suggest that AzBBr (Fig. 3C) and AzB (Fig. 4) form higher aggregates in aqueous medium and that the monobrominated derivative presents the greatest tendency for aggregation. In addition, the monomeric form of these dyes was not detected in aqueous solution.

Note that the band attributed to higher aggregate is positioned between the band corresponding to the monomer and dimer, so it is bathochromically shifted in comparison to the monomer band, but hypsochromically shifted in relation to dimer band. Although most dyes that can form different aggregates in solution have a correlative tendency between aggregation and spectral shift, it is known that the spectral shifts of different aggregates only depend on mutual orientation of components. For this reason, some compounds may form dimers (J or H) which may give rise to different tetramers (brickwork, linear or ladder assembles) with shifts toward red or blue with respect to their lowest aggregate or even their monomers [34].

As a partial conclusion, the incorporation of a bromine atom in AzB structure destabilized the J-aggregate, which could be a dimer, shifting the equilibrium toward the monomeric species in organic solvents and toward the formation of larger aggregates in aqueous solution.

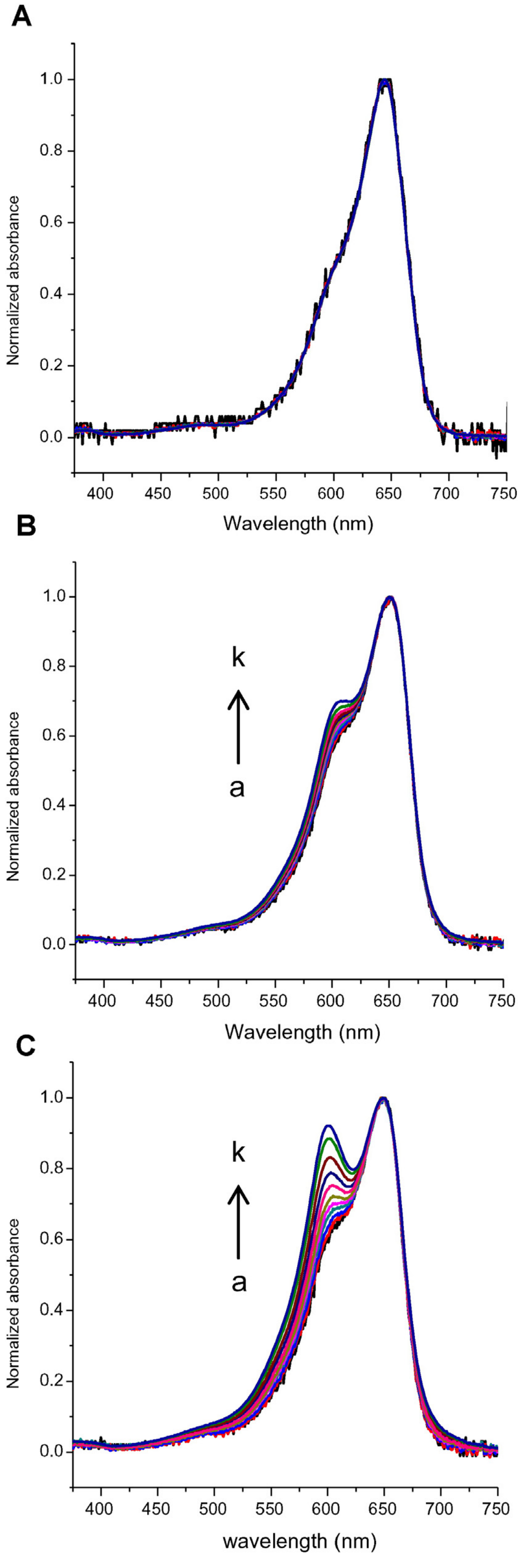


**Fig. 3.** Normalized absorption spectra of AzBBr at maximum absorption wavelength as a function of dye concentration in ethanol: water (A) 75:25 v/v, (B) 10:90 v/v and (C) pure water. [AzBBr] (a) 3.3 μM and (k) 40.2 μM.

### 3.1.3. Aggregation behavior of Th and MB in DMF and pure water

The normalized absorption spectra of AzB (Fig. 2A) and Th (Fig. 5) in DMF demonstrated that these dyes exhibit a similar behavior. In the range of concentrations assayed, these dyes showed two absorption bands: one of them, which has not been reported in literature yet and corresponds to monomeric species, was observed at approximately 500 nm, and the other one, to which we attributed the J-aggregate formation even though in the entire literature it is assigned to the monomeric species of both dyes, was seen at a wavelength >600 nm.

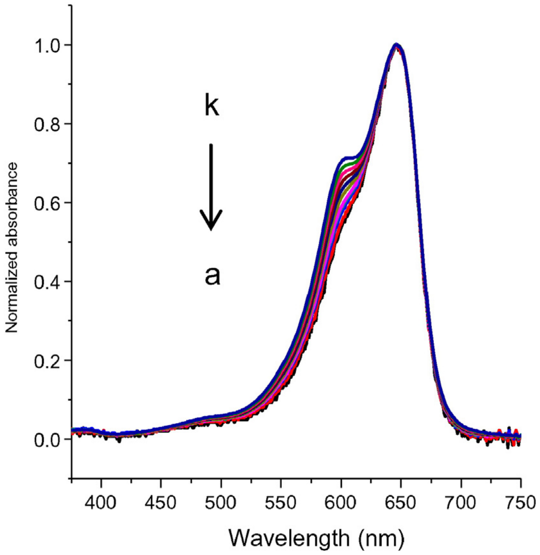


**Fig. 4.** Normalized absorption spectra of AzB in pure water. [AzB] (a) 2.0 μM and (k) 29.4 μM.

The spectra obtained for MB in DMF (Fig. 6) showed a predominant band with an absorption maximum at 666 nm, but in the normalized curves (Fig. 6, inset) it was observed that the absorbance at approximately 500 nm decreases when the dye concentration increases. In contrast to this dye, AzBBr, AzB and Th exhibited a defined band at around 500 nm. This result allowed us to infer that, like the other evaluated thiazine dyes, the monomeric form of MB presents an absorption band at approximately 500 nm.

As expected, the studies of these dyes in pure water did not exhibit the band corresponding to the monomeric species (Fig. 7). The spectra showed the bands corresponding to their respective J-aggregate. A new band at around 550–625 nm was observed when the concentration of the dyes increased, which was attributed to the formation of higher

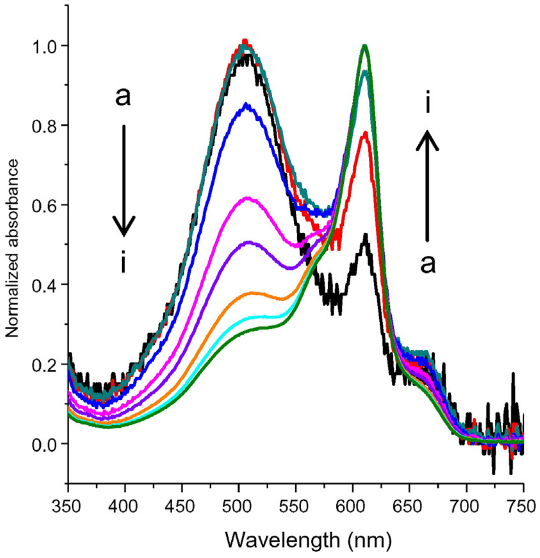


**Fig. 5.** Normalized absorption spectra of Th in DMF at maximum absorption wavelength as a function of dye concentration. [Th] (a) 1.5 μM and (i) 36.4 μM.

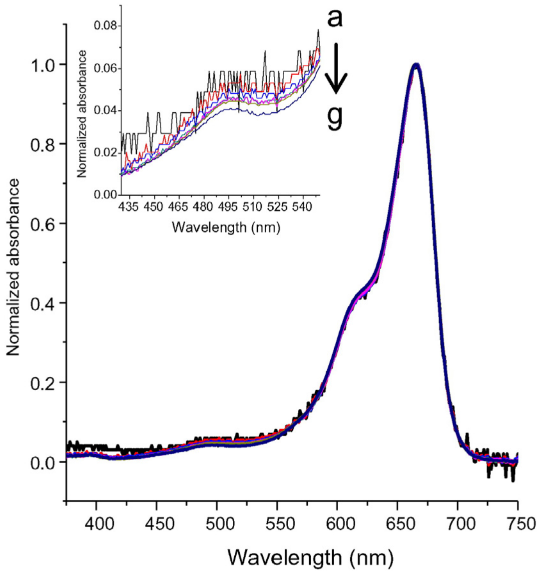


**Fig. 6.** Normalized absorption spectra of MB in DMF at maximum absorption wavelength as a function of concentration. [MB] (a) 1.3 μM and (g) 25.0 μM. Inset: Normalized absorption spectra expanded from 450 nm to 575 nm.

order aggregates. This effect was slightly observed for Th dye (Fig. 7A), while it was clearly evidenced for MB (Fig. 7B), AzB (Fig. 4) and AzBBr (Fig. 3C).

### 3.1.4. Aggregation tendency of AzBBr, AzB, Th and MB

The results obtained for all dyes in DMF (Figs. 1A, 2A, 5 and 6) show that the dimerization tendency presented the following order: MB >; Th ≈ AzB >; AzBBr. Because aggregation is a complex phenomenon, several factors should be analyzed to understand this behavior.

The MB dye includes in its structure two tertiary amines as substituents while the other thiazine dyes are substituted by primary or secondary amines at the positions $R_1$ and $R_2$ of the dye structure (Scheme 1). The hydrogen atom of these substituents might interact with solvent molecules as DMF and therefore stabilize the monomeric form. It is well known that the monomeric species are present in solution when the forces that favor the solvation of these species are stronger than dye-dye interactions [1,35]. For this reason, MB presented the highest dimerization. Furthermore, the chemical structures of Th and AzB differ in the substituents at $R_1$ and $R_2$ positions (Scheme 1). In this case, AzB dye has less possibilities to form hydrogen bonding interaction with the solvent, thus the dimerization of this dye should be higher than that of the Th dye. Besides, the methyl substituents could favor the presence of the monomeric species due to the increase in steric hindrance. These both factors contribute to similar dimerization of Th and AzB. Finally, the comparison of the results obtained for AzB and AzBBr dyes clearly shows the effect caused by bulky substituents. The substitution of a hydrogen atom for a bromine atom in the structure of AzB without modifying the hydrogen bond donor group present in the dyes destabilized the J-aggregate and contributed to the presence of the monomeric species of AzBBr.

Additionally, the self-aggregation of the thiazine dyes in water showed the following order: AzBBr > AzB ≈ MB >; Th (Figs. 3, 4 and 7). It is well known that lipophilic compounds in aqueous solutions exhibit hydrophobic repulsions with the medium, thus these compounds show greater tendency to aggregate than those of the hydrophilic derivatives [36]. As expected, the Th dye (the most hydrophilic compound) [37] presented a lower tendency for the formation of higher order aggregates, while AzBBr derivative (the most lipophilic dye) [19] showed a higher tendency for aggregation in aqueous solution. Additionally, MB and AzB dyes presented similar lipophilicity values [37,38] and showed comparable aggregation effects in an aqueous medium. These results agree with those published by Chakraborty et al. who studied the aggregation phenomenon of Th, AzB and MB among other dyes and suggested that the lipophilicity of the dye molecule increases the aggregation tendency, but in the case of MB the aggregation was smaller than expected probably due to the very high steric hindrance caused by the methyl groups [4].

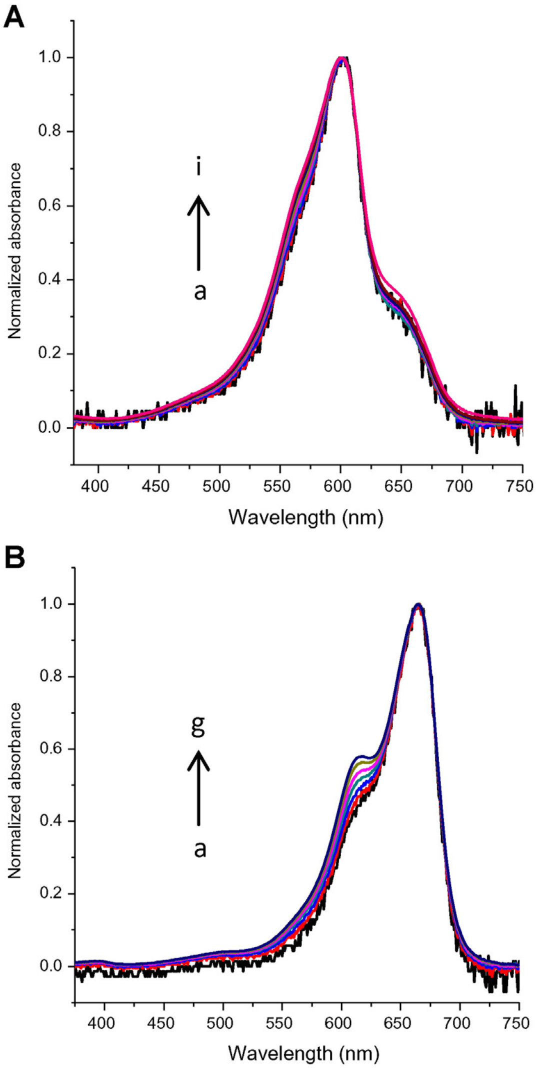


**Fig. 7.** Normalized absorption spectra in pure water of Th (A) and MB (B) at maximum absorption wavelength as a function of dye concentration. [Th] (a) 1.5 μM and (i) 80.1 μM. [MB] (a) 1.3 μM and (g) 31.3 μM.

### 3.2. Aggregation behavior as a function of temperature

It is well known that the monomeric form of numerous dyes predominates over the dimeric form when increasing the temperature [39]. In order to thoroughly evaluate the monomer-dimer equilibrium of four thiazine dyes, the impact of temperature on the monomerization of these compounds was analyzed using DMF as solvent.

The main objective of this study was to analyze the monomeric species of MB, which was predicted by a slight increase in absorbance at around 500 nm when the dye concentration decreases (Fig. 6). This effect was only observed when analyzing the normalized absorption curves of this compound in DMF.

Fig. 8 shows the spectral curves obtained for all dyes as a function of temperature. An isosbestic point was clearly observed at around 540–570 nm for all dyes. These results confirm the existence of two species in solution: monomers (500–550 nm) and dimers (600–650 nm). This

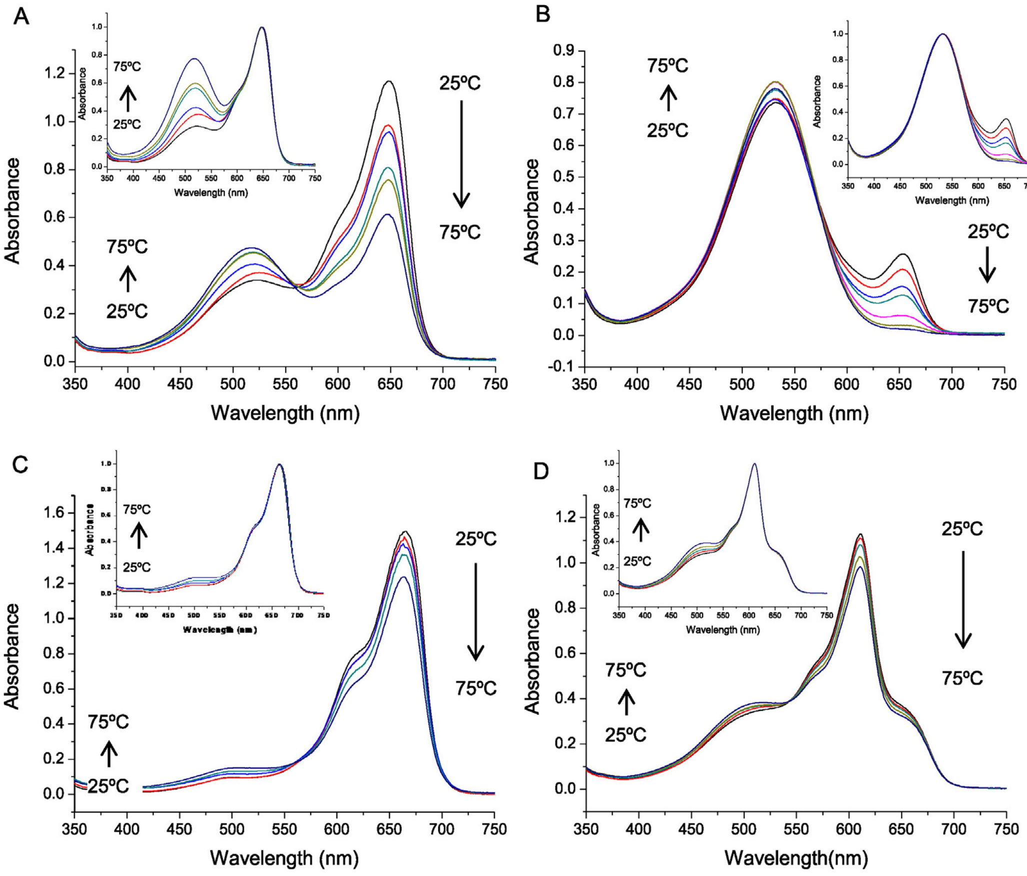


**Fig. 8.** Absorption spectra of AzB (A), AzBBr (B), MB (C) and Th (D) recorded in DMF at temperatures from 25 °C to 75 °C. Inset: Normalized absorption spectra at maximum absorption wavelength as a function of temperature.

assay was carried out at three dye concentrations and all of them showed the same tendency. For this reason, only one concentration of each dye is shown in Fig. 8. As expected, all compounds showed a decrease in the dimeric form and an increase in the monomeric species with temperature rising. In order to highlight this effect, the normalized spectral curves at the wavelength of maximum absorption of the dominant species for each dye were shown (inset of Fig. 8). The dimeric form of AzB (Fig. 8A), Th (Fig. 8C) and MB (Fig. 8D) was the principal species in solution, while the monomer was the major species for AzBBr (Fig. 8B).

These results demonstrated that the monomerization caused by temperature increase was the highest for AzBBr dye. The aggregation of this compound was completely inhibited when the sample was heated at 75 °C at all concentrations evaluated. Furthermore, the monomeric species of AzB and Th was clearly observed at three dye concentrations. Finally, the presence of MB monomer was higher with the increasing temperature (Fig. 8C) than the variations of dye concentrations (Fig. 6). These results proved that the monomeric form of MB presented an absorption maximum at around 500 nm like the other thiazine dyes. The monomerization of AzBBr, like MB, was more affected by the variations in temperature than by the variations in the concentration of the dye, while AzB and Th showed a similar behavior against both variables.

The variation in the dye concentration and changes in temperature demonstrated that the monomeric species of thiazine dyes presented an absorption maximum near 500 nm which formed J-aggregates when the dye concentration increases and temperature decreases.

All dyes proved to be stable in the experimental conditions assayed (data not shown).

### 3.3. Determination of dimerization constant

The log $K_d$ values of Th, AzB and AzBBr in DMF, at a dye concentration range from $1 \times 10^{-6}$ to $1 \times 10^{-5}$ M, were calculated using linear and nonlinear least squares curve-fitting methods. Although the monomer-dimer equilibrium of MB was evidenced under the experimental conditions, the log $K_d$ was not calculated because the monomer band was not clearly defined (Fig. 6).

The calculated monomer and dimer absorption spectra of Th, AzB and AzBBr are shown in Fig. 9. These results corroborated that the monomer-dimer equilibrium could be observed under experimental conditions because the calculated spectra of the monomeric and dimeric species showed absorption curves similar to the experimental data. The effectiveness of linear and nonlinear approaches was acceptable (data no shown) and the log $K_d$ values obtained by both methods were similar (Table 1). These results confirmed that the dimerization of Th and AzB was similar, while AzBBr showed fewer tendencies to form dimers. Besides, the substitution for bromine atom in the structure of AzB caused an increase in the maximum absorption wavelength as well as in the molar absorption coefficient ($\varepsilon$) of the monomeric and dimeric species. This effect results from the placement of a substituent with lone pairs (weakly electronegative) in a particular position in the thiazine system.

## 4. Conclusion

In this work, we corroborate that aggregation is a complex phenomenon that affects the spectrophotometric properties of the dyes. The tests carried out in organic solvents allowed the identification of monomeric species of thiazine dyes which were wrongly assigned for years.

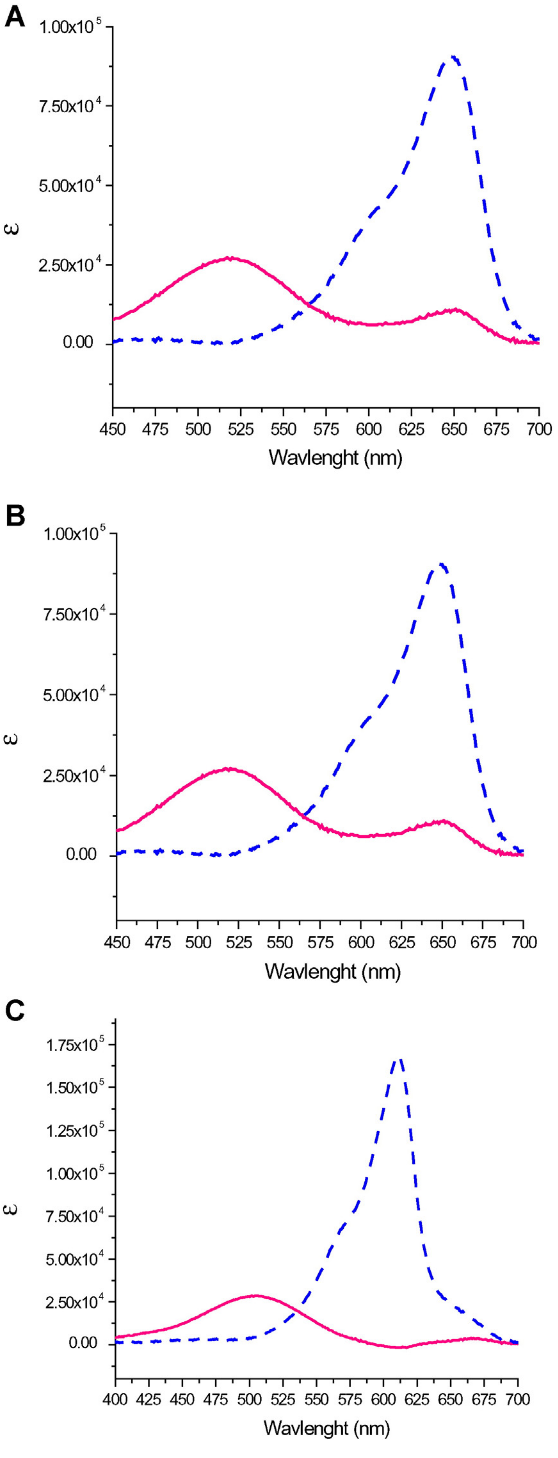


**Fig. 9.** Calculated monomer (solid line) and dimer (dash line) absorption spectra of (A) Th, (B) AzB and (C) AzBBr in DMF.

**Table 1**
Decomposition results obtained by DECOM.

| Dye | Log $K_d$ | | $\varepsilon_{Monomer}$ ($M^{-1}$ $cm^{-1}$) | $\varepsilon_{Dimer}$ ($M^{-1}$ $cm^{-1}$) |
|---|---|---|---|---|
| | Linear | Nonlinear | | |
| Th | 4.35 | 4.65 | $2.85 \times 10^4$ ($\lambda = 504$ nm) | $1.69 \times 10^5$ ($\lambda = 611$ nm) |
| AzB | 4.50 | 4.80 | $2.72 \times 10^4$ ($\lambda = 520$ nm) | $9.09 \times 10^4$ ($\lambda = 648$ nm) |
| AzBBr | 4.02 | 4.05 | $3.55 \times 10^4$ ($\lambda = 532$ nm) | $1.85 \times 10^5$ ($\lambda = 655$ nm) |

This species showed an absorption maximum between 500 and 540 nm in the used solvents. The increase in dye concentration and the decrease in temperature favored the formation of J-aggregates with maximum absorption at wavelengths >600 nm. In the literature, this species was incorrectly assigned to monomeric form of thiazine dyes. In the present work, it was demonstrated that this species corresponds to the dimeric form of AzB, AzBBr, MB and Th. In aqueous solutions, these dyes showed equilibrium between the J-aggregates and other higher order assemblies of molecules as a function of dye concentration.

In addition, the aggregation tendency of these dyes was established. The dimerization behavior presented the following order: MB > Th ≈ AzB > AzBBr, while the self-aggregation of the thiazine dyes in water was AzBBr > AzB ≈ MB > Th. Several parameters such as lipophilicity, effect of bulky substituents and interactions with the media were considered to explain the aggregation effect of these compounds in different solvents. The incorporation of a bromine atom in AzB structure destabilized the J-aggregate, shifting the equilibrium toward the monomeric species in organic solvents and toward the formation of higher aggregates in aqueous solution.

The self-association of dyes has fundamental consequences for applications as diverse as photographic technology, tunable lasers, molecular optoelectronic devices, fluorescence depolarization diagnostic devices, and photomedicine. For this reason, the correct assignment of the species in different solutions is relevant to evaluate physicochemical, photophysical and biological properties of innumerable compounds.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.saa.2016.05.005.

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