Visible Light Spectroscopic Analysis of Methylene Blue in Water; What Comes after Dimer?

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ABSTRACT: As in our previous work, most attempts to study the self-aggregation of methylene blue (MB) in water have been limited to the dimer. In the present work, we have analyzed the self-aggregation of MB in water beyond the dimeric form. For this purpose, the visible light absorption spectra of a large number of aqueous solutions of MB (1.1 × 10⁻⁶ to 3.4 × 10⁻³ M) and NaCl (0.0–0.15 M) at different temperatures (282–333 K) have been fed to a mathematical routine in order to determine the potential existence of a unique higher-order aggregate without any preconception about the aggregation order or about the need of counterions, such as chloride, for compensating the positive charge of the aggregates. Contrary to the common belief that the trimer is the dominant aggregate at high MB concentration, to our surprise we found that the tetramer acting alone, and without any counterion, is the higher-order aggregate that yields the best fitting to all the experimental absorbance spectra, with a very low average relative error of 0.04 ± 0.34%. Also contrary to previous assumptions, it has emerged quite evidently that this aggregate is present in the solution at MB concentrations below 3.4 × 10⁻⁵ M (11 ppm), though to a rather low extent. This has brought the need for the recalculation of the visible light absorption spectrum and the thermodynamic parameters for the dimer, which along with those for the tetramer are the main contributions of the present work.

1. INTRODUCTION

Methylene blue (MB) is a widely used dye in the textile industry in wool, cotton, and silk dyeing. It is also used to dye specific tissues and fluids of the body before or during surgery and diagnostic examinations, as an antiseptic and inner cicatrizier and as a staining agent for microscope analysis. On the negative side, MB accounts for a significant part of the approximately 85,000 tons of dyes and pigments that are discharged in the rivers and lakes of the world each year, affecting the freshwater aspects of the water cycle. The negative impacts of MB on human beings and animals comprise irritation of mouth, throat, esophagus, and stomach with symptoms of nausea, abdominal discomfort, vomiting, and diarrhea. Thus, the removal of MB dye from wastewater is of great concern both from a human and an environmental point of view. For this reason, MB is commonly used as a model contaminant in adsorption or photocatalytic processes. These processes may be greatly affected by the ability of the aqueous MB dye molecules to organize themselves into aggregates of different orders, depending on the total MB concentration and the temperature. Just as an example, an adsorbent that may prove efficient for adsorbing the MB monomer might, on the other hand, have a pore system unable to accommodate the aggregates. Furthermore, the evaluation of the MB concentration itself by means of spectroscopic techniques is greatly affected by the aggregation degree because all species in solution, whether or not aggregated, show fairly different optical spectra. The self-aggregation of MB in water to form a dimeric species has been researched for many decades, resulting in several scientific works in which the evaluation of the visible light absorption spectra for the monomer and the dimer, as well as the thermodynamic parameters for the equilibrium between both species (in most cases the equilibrium constant at room temperature (Kₐ) and, less often, the enthalpy and entropy of dimerization), has been obtained via extrapolation at a very low MB concentration, which along with those for the tetramer are the main contributions of the present work.

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6.3 × 10^{-7} M, shows a slight decrease at increasing temperatures. In our work, we have proven that the temperature-dependent absorption behavior of the monomer is provoked by the change in its electron charge distribution with the variation in the temperature-dependent dielectric constant of water. The monomer charge distribution stands between those of the virtual resonance forms, being the absorption spectrum of the monomer a composition of the theoretical spectra for the virtual mesomers I and II, whose proportion is established by a temperature-dependent virtual equilibrium constant, \( K_{m} \) (resonance virtual equilibrium hypothesis). At low MB concentration (below ~10 ppm) the only aggregate in solution is the dimer, whose concentration is determined by the equilibrium constant \( K_{d} \). (B) Optical spectra for the mesomers and the dimer. (C) Experimental (symbols) vs calculated (lines) absorbance spectra at different MB concentrations and temperatures.

The application of this hypothesis at MB concentrations below 3.4 × 10^{-5} M (11 ppm) allowed the thermodynamic parameters for the equilibria displayed in Figure 1A and the spectra for the dimer and mesomers (Figure 1B) to be evaluated with remarkable accuracy (Figure 1C). These spectra were obtained under the hypothesis that below 3.4 × 10^{-5} M the aggregates in solution beyond the dimer have a negligible presence, as has been often claimed. As we will prove in the present work, this is not completely precise, and though the optical and thermodynamic parameters of the mesomers remain the same as in our previous work, those of the dimer will be changing a bit.

In the present work, we have analyzed the self-aggregation of MB in water beyond the dimeric form for highly concentrated solutions. At high MB concentrations, the existence of MB trimers and higher-order aggregates in water has been assumed since long, especially when they are adsorbed on solid surfaces. According to different authors, the threshold of the trimer formation at room temperature lies in the range 10^{-5} to 5 × 10^{-5} mol L^{-1}. Since 1968, there have been a limited number of works attempting to determine the equilibrium constants for the formation of the higher-order aggregates, which are typically assumed to be trimers alone or at most accompanied by tetramers. The results of these works are summarized in Table 1.

The assumption that the higher-order aggregate at high MB concentrations is a trimer was first postulated by Braswell, who became a major referent for all the later authors. He assumed that the Debye–Hückel limiting law applies and only the cation aggregates in a MB concentration range in which the monomer and dimer are absent (0.017–0.055 M) and concluded that the limiting form of aggregation is a trimer, which is in good agreement with the conclusions arrived at from his spectral studies. However, the limiting case of the Debye–Hückel law can hardly be applied for such high MB concentration levels, unless a negligible ionic radius of the aggregate is considered, a fact that can in no way be justified. In addition, his spectral studies were biased by the use of the mean activity coefficients instead of the individual activity coefficients in the equilibrium constants.

Few of the works included in Table 1 consider the need that chloride is bonded to the trimer to compensate the excess of the...
positive charge. The inclusion of counterions in the trimer composition has been an issue in scientific discussions for decades now. From the original works by McKay and Hilson, back in 1965, the metachromatic effect of adding salts to the MB solutions became evident. However, the way the counterion acts in solution, whether by changing the aggregate structure or by affecting the activity coefficients, has never been fully clarified. The association of chloride with the aggregates is implicitly questioned from the analysis of the results provided by Rabinowitch and Epstein. Ghosh and Mukerjee also assumed the association of chloride with the trimer for their results.

Table 1. Values of Cumulative Constants for MB Aggregate Formation in Water Reported in the Literature

| Refs | Year | T (K) | $K_d \times 10^{-3}$ (L mol$^{-1}$) | $K_{3&1} \times 10^{-6}$ (L$^2$ mol$^{-2}$) | $K_{3&3} \times 10^{-10}$ (L$^3$ mol$^{-3}$) | $K_{4&0} \times 10^{-10}$ (L$^3$ mol$^{-3}$) |
|------|------|------|-----------------------------------|-------------------------------------|-----------------------------------------------|---------------------------------------------|
| 23   | 1968 | 303  | 2.00                              | 6.00                                |                                               |                                             |
| 28   | 1970 | 298  | 2.09−2.50                         | 6.25−9.40                           | 1.53−2.73                                     |                                             |
| 11   | 1975 | 300  | 2.50                              | 15.75                               |                                               |                                             |
| 22,29| 1979 | 298  | 2.54                              | 3.43                                | 0.07                                          |                                             |
| 19   | 1999 | 298  | 6.67                              | 27.93                               |                                               |                                             |
| 19   | 1999 | 298  | 6.94                              | 3.11                                |                                               |                                             |
| 21   | 2011 | 298  | 6.41$^b$                         | 3.14$^c$                           |                                               |                                             |
|      |      | 308  | 5.88                              | 2.58                                |                                               |                                             |
|      |      | 318  | 5.71                              | 2.35                                |                                               |                                             |
|      |      | 328  | 5.29                              | 2.04                                |                                               |                                             |
|      |      | 338  | 5.00                              | 1.85                                |                                               |                                             |
|      |      | 348  | 4.72                              | 1.71                                |                                               |                                             |
| this work | 2020 | 282  | 20.77$^d$                        | 953.89$^e$                         | 124.54                                        | 13.43                                        |
|      |      | 296  | 11.22                             | 13.11                               |                                               |                                             |
|      |      | 313  | 5.72                              | 13.43                               |                                               |                                             |
|      |      | 333  | 2.83                              |                                               |                                               |                                             |

Table 1. Values of Cumulative Constants for MB Aggregate Formation in Water Reported in the Literature

| Refs | Year | T (K) | $K_d \times 10^{-3}$ (L mol$^{-1}$) | $K_{3&1} \times 10^{-6}$ (L$^2$ mol$^{-2}$) | $K_{3&3} \times 10^{-10}$ (L$^3$ mol$^{-3}$) | $K_{4&0} \times 10^{-10}$ (L$^3$ mol$^{-3}$) |
|------|------|------|-----------------------------------|-------------------------------------|-----------------------------------------------|---------------------------------------------|

$K_d$: equilibrium constant of dimer formation $[2\text{MB}^+ \leftrightarrow \text{MB}_2^+]$. $K_{3&1}$: equilibrium constant of trimer formation without chloride $[3\text{MB}^+ \leftrightarrow \text{MB}_3^+]$. $K_{3&3}$: equilibrium constant of tetramer formation without chloride $[4\text{MB}^+ \leftrightarrow \text{MB}_4^+]$. $\Delta S_d$ = 6 J mol$^{-1}$ K$^{-1}$. $\Delta H_d$ = −5.2 kJ mol$^{-1}$. $\Delta S_{3&1}$ = 16 J mol$^{-1}$ K$^{-1}$, $\Delta H_{3&1}$ = −10.4 kJ mol$^{-1}$.

2. MATHEMATICAL ROUTINE

Following the resonance virtual equilibrium hypothesis and assuming a unique higher-order aggregate, formed by the association of $n$ monomers with chloride ions $[n\text{MB}^+ + n\text{Cl}^− \leftrightarrow (\text{MB}_n^+)(\text{Cl}_n^-)]$, coexisting in solution with the monomeric and dimeric forms of MB, the application of the Beer–Lambert equation at a given wavelength yields

$$A_2 = I_L \varepsilon_c \varepsilon_{\text{MB}} = A_2^{\text{ml}} + A_2^{\text{d}} + A_2^{n\text{Cl}}$$

$$= I_L (\varepsilon_c \varepsilon_{\text{ml}} + \varepsilon_c \varepsilon_{\text{d}} + \varepsilon_c \varepsilon_{n\text{Cl}})$$

$A_2$ and $A_2^{i}$ are the absorbances at a given wavelength, $I_L$ of the solution and the species, respectively, where $i$ represents each of the different MB species (m and ml: mesomers I and II; d: dimer; nCl: higher-order aggregate). The optical path length, $L$, is expressed in cm. $\varepsilon_c$ and $\varepsilon_{i}$ are the molar attenuation coefficients of the solution and the $i$ species, respectively (L mol$^{-1}$ cm$^{-1}$). $C_{\text{MB}}$ and $C_l$ are the total molar concentration of MB (expressed as monomeric units) and the molar concentration of species $i$, respectively (mol L$^{-1}$). As explained in the Experimental Section, $I_L$ is the instrumental factor.

First, the total MB and NaCl concentrations (mol L$^{-1}$) are evaluated from the MB and NaCl molalities ($m_{\text{MB}}$ and $m_{\text{NaCl}}$ mol kg$^{-1}$) and the solution density ($\rho_s$, g cm$^{-3}$), neglecting the influence of MB in the solution density

$$C_{\text{MB}} = \frac{\rho_s \times m_{\text{MB}}}{1 + 0.05844 \times m_{\text{NaCl}}}$$

$$C_{\text{NaCl}} = \frac{\rho_s \times m_{\text{NaCl}}}{1 + 0.05844 \times m_{\text{NaCl}}}$$

where $\rho_s$ is calculated for the different temperatures and salt concentrations, as indicated in the Supporting Information (Figure S1). The activity coefficients of the different ionic species in solution are evaluated by means of the equation proposed by Samson et al., which is a modification of the Davies equation to cover a wide range of ionic strength values ($I \leq 1$ mol L$^{-1}$).
\[
\ln \gamma = \frac{-4.2018 \times 10^6 Z^2_t}{(eT)^{1/2}} \sqrt{I} + \frac{0.293 \times 10^9 I}{(eT)^{1/2}} - (0.2 - 4.17 \times 10^{-12} I) I
\]  

(3)

In this equation, \(Z_t\) and \(e\) are the electronic charge and the ionic radius \((m)\) of species \(i\), \(T\) is the solution temperature \((K)\), \(I\) is the ionic strength \((\text{mol L}^{-1})\), and \(e\) is the relative permittivity \((\text{dielectric constant})\) of water. From values reported in the literature, \(^37\) the following \(\varepsilon_r\) versus \(T\) relationship was found

\[
\varepsilon_r = 87.9 - 0.4002ST + 7.625 \times 10^{-14}T^2
\]  

(4)

The ionic radii for chloride and sodium ions take values of \(2 \times 10^{-10}\) and \(3 \times 10^{-10}\) \(m\), respectively. \(^35\) For the MB ions, approximate radii can be calculated considering the dimensions of the MB molecule \((17.0 \times 7.6 \times 3.3 \AA)\). \(^22,38\) For the sake of simplicity, we assume that an aggregate is a simple stack of MB molecules and its ionic radius is that of the sphere with the same volume as the stack. This simplified picture yields the ionic radius of a MB aggregate formed by \(n\) monomers as

\[
r_i = 4.67 \times 10^{-10} n^{1/3}
\]  

(5)

With this equation, the ionic radii for the monomer, dimer, trimer, and tetramer are estimated to be \(4.7 \times 10^{-10}\), \(5.9 \times 10^{-10}\), \(6.7 \times 10^{-10}\), and \(7.4 \times 10^{-10}\) \(m\), respectively.

Considering the mass and charge balances and neglecting the influence of hydrogen and hydroxyl ions, the ionic strength can be evaluated as follows

\[
I = 0.5 \times (3C_{\text{MB}} - C_{\text{mll}}(1 + K_m) + 2C_{\text{NaCl}} + [n^2 - n(2c + 2) + c(c - 1)]C_{n_{\text{kc}}})
\]  

(6)

where \(K_m\) is the dimensionless equilibrium constant for the virtual equilibrium between mesomeric forms \(^8\)

\[
K_m = \frac{C_{\text{mll}}}{C_{n_{\text{kc}}}} = e^{(\Delta S_m/R - \Delta H_m/RT)}
\]  

(7)

The values for \(\Delta S_m\) and \(\Delta H_m\) are indicated in Figure 1. Similar equations can be introduced for the cumulative formation constants of the dimer and the higher-order aggregate

\[
K_d = \frac{\gamma_d C_d}{\gamma_m(C_{\text{mll}} + C_{n_{\text{kc}}})^2} = e^{(\Delta S_d/R - \Delta H_d/RT)}
\]  

(8)

\[
K_{n_{\text{kc}}} = \frac{\gamma_{n_{\text{kc}}} C_{n_{\text{kc}}}}{\gamma_m C_{\text{cI}}^n (C_{\text{mll}} + C_{n_{\text{kc}}})^n C_{\text{cI}}^c} = e^{(\Delta S_{n_{\text{kc}}}/R - \Delta H_{n_{\text{kc}}}/RT)}
\]  

(9)

where \(C_{\text{cI}}\) is the chloride concentration \((\text{mol L}^{-1})\) and \(K_d\) \((\text{L mol}^{-1})\) and \(K_{n_{\text{kc}}}\) \((L^{n+c-1} \text{ mol}^{n-c})\) are the equilibrium constants for the formation of the dimer and the higher-order aggregate.

From the mass and charge balances and the equilibrium constants, the concentration of the mesomers and the dimer can be expressed as

\[
C_{\text{mll}} = K_m C_{\text{mll}}
\]  

(10)

\[
C_{n_{\text{kc}}} = C_{n_{\text{kc}}}^* = \frac{E(C_{\text{mll}}^* C_{n_{\text{kc}}})}{E(C_{\text{mll}}^* C_{n_{\text{kc}}})}
\]  

(15)

where \(C_{n_{\text{kc}}}^*\) and \(C_{n_{\text{kc}}}^*\) come from steps \#1 and \#2, respectively, and \(E(C_{\text{mll}}^* C_{n_{\text{kc}}})\) is the derivative of \(E(C_{\text{mll}} C_{n_{\text{kc}}})\) with respect to \(C_{n_{\text{kc}}}\) evaluated at \(C_{n_{\text{kc}}}^*\) and \(C_{n_{\text{kc}}}^*\). This derivative is calculated as
where  

\[ h = e^{z_1 - z_2} \]  

\[ z_1 = 2 \ln c + \frac{n}{c} \times \ln[C^*_{\text{nill}}(1 + K_m)] + \ln(\gamma_c) \]  

\[ z_2 = \frac{1}{c} \times [\ln(\gamma_{\text{nc}}) + \ln(C^*_{\text{nc}}) - \ln(K_{\text{nc}})] \]  

To derive eq 16, it was assumed that \( \frac{\partial \gamma_c}{\partial C_{\text{nc}}} \approx 0 \), which is essentially true at high ionic strength values. The evaluation of \( h \) must be performed as indicated above to avoid overflow errors.

Finally, \([\#6]\) \( \gamma_c \) and the new \( C_{\text{nc}} \) value are fed to step \#2 and a new iteration is performed within the function. The convergence

Table 2. Optical and Thermodynamic Parameters of MB in Water

| species          | \( \lambda_{\text{max}} \) (nm) | \( \varepsilon_{\text{max}} \times 10^{-4} \) | L mol\(^{-1}\) cm\(^{-1}\) |
|------------------|---------------------------------|---------------------------------------------|
| Tetramer         | 600                             | 13.85                                       |
| Dimer            | 607                             | 10.14                                       |
| monomer (mesomer II) | 664                           | 10.78                                       |
| monomer (mesomer I) | 650                           | 4.40                                        |

| thermodynamic parameters          | Monomer ↔ Tetramer | Monomer ↔ Dimer | Mesomer II ↔ Mesomer I |
|-----------------------------------|--------------------|----------------|------------------------|
| \( \Delta S_{\text{tet}} \) (J mol\(^{-1}\) K\(^{-1}\)) | -109.4          | -100.9         |                        |
| \( \Delta H_{\text{tet}} \) (kJ mol\(^{-1}\)) |                      |                |                        |
| \( \Delta S_{\text{d}} \) (J mol\(^{-1}\) K\(^{-1}\)) | -25.6            | -30.5          |                        |
| \( \Delta H_{\text{d}} \) (kJ mol\(^{-1}\)) |                      |                |                        |
| \( \Delta S_{\text{m}} \) (J mol\(^{-1}\) K\(^{-1}\)) | 24.0             |                |                        |
| \( \Delta H_{\text{m}} \) (kJ mol\(^{-1}\)) |                      |                |                        |

Figure 2. Relative errors (upper plots) and total errors (lower plots) obtained for different values of \( n \) and \( c \). (A) \( n & c \) scenario; (B) \( m/d & n & c \) scenario; and (C) \( d/n & c \) scenario.

Figure 3. Absorption spectra for the mesomers I and II evaluated in both the \( m/d & n & c \) scenario (c,a) and the \( n & c \) scenario (b,d).

Figure 4. Optical spectra for all the MB species either virtually (mesomers) or truly contained in aqueous solutions.
was considered sufficient when the relative difference of values fed into and obtained from eq 15 was below $10^{-4\%}$. The mass and charge balances were used to prove the viability of the function.

With the thermodynamic values obtained by means of the Solver tool in combination with the Excel function described above, the rest of attenuation coefficients needed to fill the spectrum in the whole wavelength range (500–700 nm) were obtained by repeating the routine at each wavelength value, in a process that was automated by a number of Excel macros.

For a more complete understanding of the goodness of fit, the average relative error (%) was evaluated as

$$
\langle E_i \rangle = \frac{100}{224 \times 7} \sum_{i=1}^{224} \left( \frac{A_{\lambda_{i,exp}} - A_{\lambda_{i,cal}}}{A_{\lambda_{i,exp}}} \right)
$$

(20)

Finally, the molar fractions of the different species in solution were evaluated as

$$
X_{ni} = \frac{C_{ni}}{C_{MB}}, \quad X_{nii} = \frac{C_{nii}}{C_{MB}}, \quad X_d = \frac{2C_2}{C_{MB}},
$$

(21)

3. RESULTS AND DISCUSSION

Three different scenarios have been analyzed: (A) the optical and thermodynamic parameters for the mesomers and the dimer are set to those evaluated in our former work (Figure 1) and only the parameters of the higher-order aggregate are varied to minimize the total error (hereafter called the "n&c scenario"); (B) the optical and thermodynamic parameters for all MB...
species in solution are varied (hereafter called the "m/d/n&c scenario") and (C) the optical and thermodynamic parameters for the mesomers are set to those evaluated in our former work (Figure 1) and the parameters of both the dimer and the higher-order aggregate are varied (hereafter called the "d/n&c scenario"). Figure 2 shows the errors obtained in the three scenarios, for different values of n and c.

In all cases, it was found that integer values of c over 1 (more than one chloride anion linked to the higher-order aggregate) produced significantly higher errors than those obtained for c = 0 or c = 1, so that the corresponding solutions were automatically dismissed. The n&c scenario yielded the errors displayed in Figure 2A. The best $\langle E_T \rangle$ value was obtained by considering that the higher-order aggregate is a tetramer without chloride, $n = 4$ and $c = 0$ (or 4&0), although the parameters obtained with this solution were dismissed for the following reasons: (i) the total error was significantly higher than those obtained in the other scenarios, (ii) the standard deviation of the relative error was too high to comply with a stringent standard of goodness of fit and reflected a trend to underestimate the values of absorbance $\langle E_r \rangle = 1.81 \pm 2.75\%$, and (iii) the calculated fraction of tetramer ($X_{4&0}$) for the solution with 11 ppm of MB and no NaCl at 282 K was as high as 0.08, conflicting with our previous assumptions, which included the absence of a higher-order aggregate at $C_{MB}$ below or equal to 11 ppm. Therefore, it must be assumed that some changes in the previously reported visible light absorption spectra must be introduced. In that work, the values of the activity coefficients were considered to be always one. In the

Figure 6. Experimental (crosses) and calculated (lines) absorbance curves for the solutions prepared at low MB concentrations and different temperatures in the presence of NaCl.
absence of a higher order aggregate, this approximation is almost exact for $C_{\text{NaCl}} = 0$ M because the lowest value for the dimer activity coefficient evaluated with eq 3 is 0.97. Thus, the $m/d/n&c$ scenario, in which the attenuation coefficients and the thermodynamic parameters for all the species in solution are simultaneously evaluated, should provide either a different set of parameters for the mesomers and the dimer if the higher-order aggregate is present at a low concentration ($C_{\text{MB}} \leq 11$ ppm and $C_{\text{NaCl}} = 0$ M) or very similar spectra to those previously reported if the higher-order aggregate is absent at such a concentration range. Any other combination should be considered the result of either a chaotic fit or the noncompliance with the model premises (a unique higher-order aggregate). The fitting process under the $m/d/n&c$ scenario yielded the errors, as displayed in Figure 2B. Interestingly, the lowest $\langle E_r \rangle$ error was obtained by considering that the higher-order aggregate is a hexamer with one chloride anion ($6&1$). This error was also the lowest of all the scenarios. However, the relative error (upper plot in Figure 2B) still involved a significant standard deviation, though now with a certain overestimation of the absorbance values ($\langle E_r \rangle = -0.55 \pm 0.83\%$). Nevertheless, the reason that leads us, without a doubt, to dismiss this solution is the fact that even though the calculated fraction of the hexamer ($X_{6&1}$) for the solution with 11 ppm of MB and no NaCl at 282 K was almost 0 ($X_{6&1} = 0.001$), the new absorption spectra for the mesomers were very different from those previously reported (Figure 3). In fact, the error in the low concentration zone ($C_{\text{MB}} \leq 11$ ppm and $C_{\text{NaCl}} = 0$ M) for the $m/d/n&c$ scenario was

Figure 7. Experimental (crosses) and calculated (lines) absorbance curves for the solutions prepared at high MB concentrations and different temperatures in the presence of NaCl.
considerably higher than the error for the n&c scenario (2.4 × 10⁻⁴ vs 1.8 × 10⁻⁴).

Thus, either the unique higher-order aggregate premise is incorrect or the m/d/n&c scenario produces a chaotic fit. To check this second option, we have used a rational approach by which the optical spectra and the thermodynamic parameters for the mesomers, reported in our previous work, were considered to be correct and the parameters of both the dimer and the higher-order aggregate were varied (d/n&c scenario). This scenario is well in tune with the long-accepted principle that the extrapolation methods give good results with the monomer but are less reliable with respect to the dimer.10,12,31 In fact, by applying

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**Figure 8.** Variation of the molar fractions of the monomer, mesomer I, mesomer II, dimer, and tetramer with the MB concentration at different temperatures. (A) C_{NaCl} = 0 M and (B) C_{NaCl} = 0.15 M.

**Figure 9.** Variation of the activity coefficients with the ionic strength for the different species (the subscript DH refers to the values evaluated with the Debye–Hückel limiting law).
Figure 10. Application of eq 1 with the optical and thermodynamic parameters obtained in this work to the experimental data obtained by Braswell23 $[\Delta\lambda = -2.0\ \text{nm}, I_p = 1.157\ (A, B)\ 1.359\ (C)]$.

Figure 1 shows the conspicuous increase of the shoulder at $\lambda = 660\ \text{nm}$ to become a peak by its own merit. The tetramer has a single maximum at 600 nm and is responsible for the blue shift at high values of the ionic strength. All the optical and thermodynamic parameters for the monomer and the aggregates are summarized in Table 2. The new thermodynamic parameters of the dimer do not differ substantially from those evaluated in our previous work.$^8$

With respect to the tetramer, the negative increment of enthalpy indicates, as in the case of the dimer, that aggregation is an exothermic process, whereas the negative entropy change is because of the association of similarly charged species,$^{39}$ which might be more reasonable than the positive values found by Klika et al.22 Figures 5-7 show the experimental and calculated values of absorbance for all the solutions analyzed in this work. Beyond the low value of the relative error, these figures provide visual proof of the goodness of fit, which can be considered more than satisfactory.

The molar fractions of the different species in solution were evaluated with eq 21 for different temperatures and NaCl concentrations in the $1 \times 10^{-6}$ to $3.5 \times 10^{-3}\ \text{mol L}^{-1}$ range of the MB concentration. The results are shown in Figure 8. The increase in the aggregation level with the decrease of the temperature or with the increment of the NaCl concentration is conspicuous. As observed in the figure, for $C_{\text{MB}} = 3.5 \times 10^{-3}\ \text{M}$ and $C_{\text{NaCl}} = 0.15\ \text{M}$ most of the MB molecules in solution are associated as tetramers $(X_{4\&0} = 0.93)$. As commented in the Introduction section, Braswell$^{23}$ proceeded from the assumption that the Debye–Hückel limiting law is applicable to prove that the limiting form of aggregation is a trimmer. As observed in Figure 9, the activity coefficients evaluated by eq 3 are comparable to those calculated with the Debye–Hückel limiting law only in the case of the monomer and, in such cases, only at low ionic strength values. It should be emphasized that these coefficients are raised to the positive integer exponents in the expressions for the equilibrium constants [eqs 8 and 9], so that the differences observed in Figure 9 are in fact being magnified in such expressions. The effect of adding salt on the activity coefficients is evident from the results shown in the figure. This ends the argument about the real effect of chloride on the
MB aggregation, proving correct the theory that favors the variation in the activity coefficients over the structural changes in the agglomerates as the consequence of adding salt, at least in the concentration ranges studied in this work.

Finally, we have applied the optical (Figure 4) and thermodynamic (Table 2) parameters obtained in this work to a number of absorbance spectra reported in some of the oldest and most cited works dealing with the phenomenon of MB aggregation.23,30 Their authors used optical cells made of different materials and with different path lengths, and in one case achieved with the help of spacers.30 The variability of cells results in unavoidable differences among spectra measured at the same concentration with different cuvettes. Added to this, there is a known wavelength sensitivity in the equipment used before 1975 that is assumed to be around ±3 nm.40 These two issues were accounted for in the application of eq 1 to the experimental data by means of two parameters. The first is the instrumental factor, \( I_F \), that eliminates differences between cells. The second parameter is the difference in sensitivity between the equipment used in this work and those employed in the works referred above, expressed as a shift in the wavelength (\( \Delta \lambda \)).

As commented in the Experimental Section, at a high NaCl concentration (0.9 M) the extensive precipitation of MB aggregates took place (Figure S2). This is consistent with the significant blueshift in the absorption spectrum that McKay and Hillson30 found for solutions at \( C_{NaCl} = 0.9 \) M, although these authors did not report the precipitation of MB at such conditions. This blueshift could not be reproduced with the attenuation coefficients and thermodynamic parameters obtained in this work (Figure 14) with the same level of precision reached for lower NaCl concentrations (Figure 13).

As the activity coefficients are independent of the ionic strength for values of \( I_F \) higher than around 0.1 M (Figure 9), it seems evident that at very high chloride concentrations, the Cl\(^-\) anions must participate in the formation of over-aggregates of MB, possibly by linking together tetramers, which ultimately

**Figure 11.** Application of eq 1 with the optical and thermodynamic parameters obtained in this work to the experimental data obtained by Ghosh11 (\( \Delta \lambda = -0.7 \) nm, \( I_F = 1.164 \) (A–C) 1.435 (D,E)).
precipitate. However, this phenomenon does not occur in the MB/NaCl concentration and temperature ranges analyzed in this work.

4. CONCLUSIONS

The visible light absorption spectra of a large number of aqueous solutions of MB (1.1 \times 10^{-6} to 3.4 \times 10^{-3} M) and NaCl (0.0–0.15 M) at different temperatures (282–333 K) have been fed to a mathematical routine in order to determine the potential existence of a unique higher-order MB aggregate without any preconception about the aggregation order or about the need of counterions such as chloride for compensating the positive charge of the aggregates. The routine considers the nonideality of the solutions in the calculation of the activity coefficients. From the analysis of different scenarios, it was found that the tetramer acting alone and without any counterion is the higher-order aggregate that yields the best fitting to all the experimental absorbance spectra, with a very low average relative error of 0.04 \pm 0.34%. In the absence of NaCl, this aggregate is present in solution at MB concentrations below 3.4 \times 10^{-5} M (11 ppm),

Figure 12. Application of eq 1 with the optical and thermodynamic parameters obtained in this work to the experimental data obtained by Bergmann and O’Konski12 [\Delta \lambda = -0.7 \text{ nm}, I_F = 1.220 (A–F) 1.281 (G,H)].
though to a rather low extent. Due to this fact, the visible light absorption spectrum and the thermodynamic parameters for the dimer had to be recalculated with respect to those evaluated in our previous work.\textsuperscript{8} The goodness of fit has been shown to be rather satisfactory by comparing experimental and calculated light absorption spectra obtained both in this work and from the literature.

5. EXPERIMENTAL SECTION

The absorption spectra (400–800 nm at 1 nm step) of different MB (C.I. 52015; analytical grade) solutions in deionized water were measured at temperatures in the 282–333 K range using a Shimadzu UV–vis spectrometer. The temperature of the optical cuvettes was kept constant using a LAUDA Alpha RA8 thermo-circulating bath. Every measurement was repeated thrice, with exhaustive cleaning of the cuvettes (water, ethanol and air drying) between measures. Four salt concentrations were employed, 0.00, 0.05, 0.10, and 0.15 mol L$^{-1}$. The solutions with MB concentrations in the $1.1 \times 10^{-6}$ to $3.4 \times 10^{-5}$ mol L$^{-1}$ (0.35–11 ppm) range were poured into UV quartz cuvettes of 700 μL volume and 1 cm path length. At higher MB concentrations ($9.4 \times 10^{-5}$ to $3.4 \times 10^{-3}$ mol L$^{-1}$ (30–1100 ppm)), the visible light absorption spectra were obtained using a flow-through UV quartz cuvette of 6 μL volume and 0.01 cm path length. The MB and NaCl concentration ranges were selected to avoid deficiencies in the absorbance measurements due to the presence of the dispersed particles and/or precipitates formed through the over-aggregation of MB molecules. As shown in Figure S2, specific solutions prepared at a high NaCl concentration (0.9 M) suffered from extensive precipitation. Before analysis, all solutions were allowed to stabilize under magnetic stirring in complete darkness overnight. A total of three optical cuvettes were used for all the analyses (two cuvettes of 1 cm and one cuvette of 0.01 cm). To take account of small variations in
the quartz transmittance and path lengths with respect to the nominal values, an instrumental factor, $I_F$, was evaluated for each cuvette so that the molar attenuation factors evaluated with a given solution were independent of the cuvette used for the cuvette so that the molar attenuation factors evaluated with a given solution were independent of the cuvette used for the.

![Graph](image)

**Figure 14.** Application of eq 1 with the optical and thermodynamic parameters obtained in this work to the experimental data obtained by McKay and Hillson at $C_{\text{NaCl}} = 0.9 \text{ M}$ ($\Delta \lambda = -1.3 \text{ nm}, I_F = 1.149$ (A) 1.255 (B,C); equal to the values in Figure 13).

graphs by means of a Visual Basic program fed with bmp-formatted scanned images of the graphs.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c03830.

Density of NaCl solutions and images of precipitated MB aggregates (PDF)

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### Notes

The authors declare no competing financial interest.

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