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Small aggregates of benzoporphyrin molecules observed in water–organic solvent mixtures

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ABSTRACT: Benzoporphyrins, chlorin-type compounds, are efficient new drugs in photodynamic therapy. In this work, some aspects of the benzoporphyrin-1,3-diene dimethyl ester (DiesterB) aggregation were investigated in water–organic solvent mixtures, using visible spectrophotometry. The effects of solvent mixtures, water with acetonitrile, dioxane, methanol and ethanol, were compared taking the calculated aggregation equilibrium constant (K) and the aggregation number. The K dependence on temperature, DiesterB concentration and water content suggest that, in water with acetonitrile, the predominant species is a dimer, whereas in dioxane, methanol and ethanol systems, there are multiple equilibria, with a pre-equilibrium between the monomer and dimer (small amount), followed by trimer formation. Not only the solvent polarity but also the nature of the organic solvent are important for the aggregation process, driving the aggregate size and the magnitude of the equilibrium constant. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: photodynamic therapy; benzoporphyrins; aggregation; homogeneous solutions; solvent effect

INTRODUCTION

Research in photodynamic therapy (PDT) has indicated porphyrins (including porphyrin-based compounds, such as purpurins, chlorins, chlorophylls and bacteriochlorins) and phthalocyanines as the most promising new photosensitizers for medical applications.[1–3] The latest medication for PDT approved by the US Food and Drug Administration (FDA), Visudyne, has been used to fight age-related macular degeneration (AMD), an eye disease which is the leading cause of blindness in the elderly.[4–6] Additionally, Visudyne active compound, named Verteporfin (BPDMA), has been tested against cancer, arthritis, autoimmune disorders, viral infections, psoriasis, herpes, bacterial control, pathological myopia, etc.[1,3,4,7] BPDMA, a benzoporphyrin monoacid chlorine-like photosensitizer, has a cyclohexadiene ring fused at a reduced pyrrole ring, ring A of the tetrapyrrol porphyrin structure, and one propyl carboxylic acid and one propyl ester group at rings C and D of the porphyrin, respectively.

Reports on some structural analogues of BPDMA which have a cyclohexadiene ring fused at reduced ring A or B (here named A-ring and B-ring derivatives, respectively) have shown that they exhibit very similar properties; for example, both analogues have the main Q band (near 690 nm) with similar molar absorptivity ($34\,000\,\mathrm{l\,mol^{-1}\,cm^{-1}}$) in dimethyl sulfoxide (DMSO), low solubility in aqueous media, high selectivity to tumour tissues and efficient singlet oxygen generation under proper wavelength irradiation.[2,5] Photosensitizing activity studies demonstrated that the position of the fused cyclohexadiene group does not affect considerably their *in vitro* efficiency[2] and that they show similar patterns of biodistribution and clearance in mice. Some small differences detected in animal studies were attributed to events occurring at the cellular level.[2]

Additionally, B-ring derivatives are more lipophilic than A-ring compounds. The high hydrophobicity of the former would enhance the association with cell membranes with an advantage for tumour cell uptake.[5] However, it is well known that hydrophobic porphyrins undergo aggregation processes in water-rich media.[8,9] Dimers and other aggregates in general exhibit low singlet oxygen yield, cancelling their PDT therapeutic effectiveness.[10,11a]

In recent work,[9] a B-ring type of photosensitizer was investigated, a tetraester porphyrin (without carboxylic acid substituents), here named DiesterB; this molecule is uncharged in neutral aqueous media and has high hydrophobic characteristics, which lead to self-aggregation in a process that can be induced simply by changing the composition of the water–organic solvent mixture. Knowledge of the mechanism of DiesterB aggregation in these solvent mixtures would permit the development of topical formulates which can be applied in PDT to superficial diseases.

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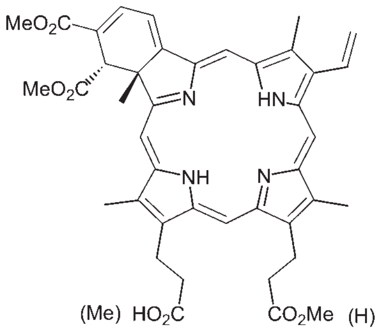
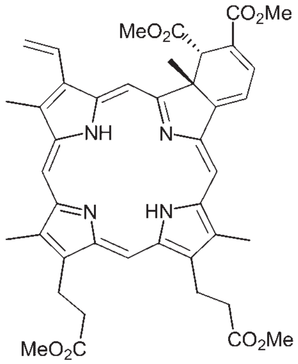


BPDMA



DiesterB

The monomeric form of DiesterB has the characteristic Q band at 690 nm whereas the aggregate exhibits a new band in the 720–740 nm region, with increased intensity as a consequence of aggregation, when the water content in water–DMSO solvent increases. It was also pointed out that the molecule aggregates as dimers.[9] In spite of this investigation, there have been no studies on the aggregation process of DiesterB in other solvent mixtures.

The aim of the present work was to extend DiesterB aggregation studies using solvents such as water with acetonitrile, dioxane, methanol and ethanol. The dependence on the water content in the mixtures, the drug concentration and temperature effects on the aggregation process of DiesterB were compared among the solvents investigated. We postulate that the aggregation process in some solvent mixtures can involve only monomers and dimers, whereas higher aggregation states must be present in other water–organic solvent mixtures.

EXPERIMENTAL

All solvents were of HPLC or spectrophotometric grade. Deionised, doubly distilled water from an all-glass apparatus was used throughout. DiesterB, synthesized according to the described method,[12] was supplied by Professor D. Dolphin (University of British Columbia, Vancouver, Canada). Stock solutions of porphyrin were prepared in DMSO (Mallinckrodt) and kept frozen in the dark. Before use, the melted solutions were standardized by UV–visible spectrophotometry, using a value for the molar absorptivity[9] of $\varepsilon = 3.4 \times 10^4\,\mathrm{l\,mol^{-1}\,cm^{-1}}$ at 690 nm. Solvent mixtures were prepared adding specific volumes of acetonitrile (Mallinckrodt), 1,4-dioxane (Merck), methanol (Aldrich) and ethanol (Merck) to volumetric flasks and diluting to volume with water. After reaching the temperature of the solvent in the cuvette, the porphyrin (DMSO stock solution) was introduced with a microsyringe followed by vigorous agitation (mechanical plate mixer, during 10 s). The final solution contained not more than 0.07% (v/v) DMSO. In water–acetonitrile solvents, the aggregation of DiesterB was observed just after mixing, as monitored by spectrophotometry, whereas for dioxane, methanol and ethanol, 20–30 min were needed to reach equilibrium.

Equipment

Spectrophotometric determinations were performed with both Cary-50 (Varian) and DU-70 (Beckman) spectrophotometers using quartz or polystyrene cuvettes with a 1.00 cm optical length and controlled temperature. For dilute solutions, a special glass cuvette with a 9.50 cm optical length was employed. The absorption spectra were recorded in the range 400–800 nm, in order to follow the consumption of monomers (Q and Soret bands decreasing) and the appearance of aggregates, as seen by an increase in absorption at 720–740 nm.

RESULTS AND DISCUSSION

In all pure organic solvents employed, the spectra of dilute solution of DiesterB exhibit two main bands, attributed to the monomer, at 690 nm (Q band) and at 430 nm (Soret band). As a consequence of the introduction of water into the organic solvent, both of the above bands undergo a decrease and a new band, near 720–740 nm, appears. Figure 1 illustrates the absorption spectra of a solution of fixed porphyrin concentration as the percentage of water in the organic solvent is increased.

One can see that the monomer peaks continuously decrease whereas the aggregate band (740 nm) increases as a function of water content (Fig. 1). An isosbestic point (at 703 nm) is observed, indicating a simple one-step equilibrium.[11b,13,14] Similar results were verified with DiesterB in water–DMSO solvent, and were attributed to unique equilibrium between the monomer (M) and the dimer (D).[9]

However, for the same experiment in water–dioxane mixtures, the isosbestic point is not so clear, and for the water–methanol and water–ethanol systems, the absence of this point is noted (it is observed as

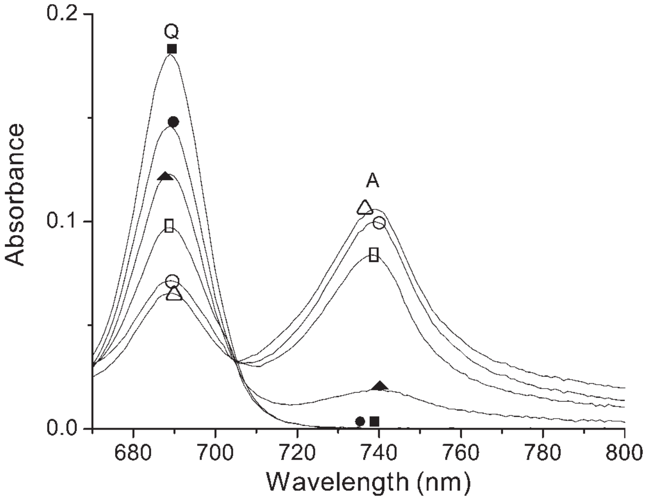


Figure 1. Absorption spectra of DiesterB, $5.37 \times 10^{-6}\,\mathrm{mol\,l^{-1}}$, at 30.0 °C with increasing amounts of water in acetonitrile mixtures. Water content: (■) 0; (•) 55; (▲) 58; (□) 60; (○) 62; (△) 65%

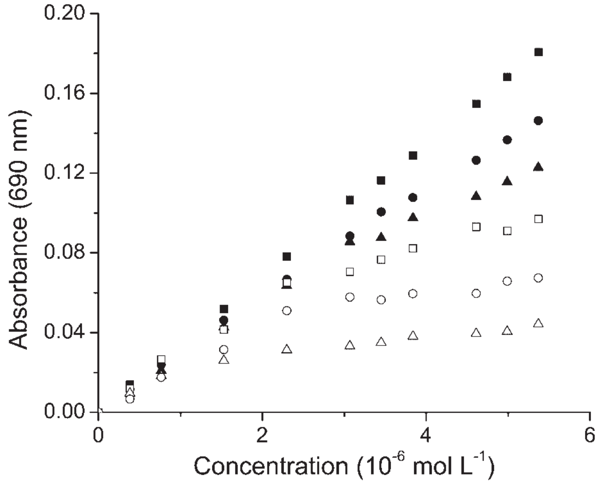


Figure 2. Absorbance behaviour at the monomer maximum versus [DiesterB] with different mixtures of water in acetonitrile at 30.0 °C. Water content: (■); 0; (•) 55; (▲) 58; (□) 60; (○) 62; (△) 65%

quasi-isosbestic). The lack of a clear isosbestic point in these solvents suggested the co-existence of other species beyond dimers and monomers.

Effect of concentration

In addition to the assays with varying water content and a fixed concentration of DiesterB, we performed experiments in which the concentration of porphyrin was increased at a fixed solvent composition, for water in different organic solvents. The overlay of the apparent absorptivity spectra (ε = absorbance/[DiesterB]) of DiesterB at a fixed 62% water–acetonitrile solvent composition, and different porphyrin concentrations shows a reduction in the monomer peak whereas the aggregate peak (740 nm) is enhanced as [DiesterB] is increased. Once again, for this solvent, an isosbestic point is observed at 703 nm. Qualitatively the same behaviour was obtained for water with dioxane, methanol and ethanol mixtures with the aggregate band positioned at around 735 nm. However, in these three solvent systems, all the spectra cross in the 703–708 nm region (not a unique wavelength), which means that a clear isosbestic point was not evidenced.

The absorbance of the monomer at a fixed wavelength as a function of [DiesterB] was analysed for pure organic solvents and their mixtures with water. In all the pure organic solvent solutions, the absorbance of the monomer at 690 nm obeys Beer's law. The molar absorptivities for acetonitrile, dioxane, methanol and ethanol were 3.4×10^4, 3.8×10^4, 3.3×10^4 and $3.4 \times 10^4\,\mathrm{l\,mol^{-1}\,cm^{-1}}$, respectively. For the aqueous mixtures, Beer's law was also verified for restricted contents of water, in such a way that the aggregate band is not observed. In acetonitrile, for instance, in the measured range up to $2.3 \times 10^{-5}\,\mathrm{mol\,l^{-1}}$ of DiesterB and up to 50% of water, all the curves exhibited similar slopes (i.e. the same molar absorptivity).

However, when the water content was increased above 50% in acetonitrile, a negative deviation was observed. This effect was more pronounced as the porphyrin concentration increased (Fig. 2).

This deviation reflects the formation of aggregates, and the presence of an isosbestic point suggests a simple one-step equilibrium, as similarly assumed by Delmarre *et al.*,[9] for DiesterB in water–DMSO solvent:

$$2\,\mathrm{M} \rightleftharpoons \mathrm{D}$$

Following the previously employed mathematical treatment,[9] the experimental data for absorbance against [DiesterB] were fitted by the following equation, allowing the calculation of the dimer equilibrium constant (K_d):

$$\mathrm{Abs_M} = \{(1 + 8\,K_d[\mathrm{Po}])^{1/2} - 1\}\varepsilon_M/(4\,K_d) \qquad (1)$$

where $\mathrm{Abs_M}$ is the absorbance at the monomer peak, ε_M is the monomer molar absorptivity and [Po] is the total porphyrin concentration. In this methodology, two statements were assumed: the overlap between the peaks of the dimer and the monomer is negligible, and ε_M does not change with water content in the solvent. The former statement is true for the peak at 690 nm where only monomer absorbs, whereas for the latter, ε_M used here is the value obtained from experiments in pure acetonitrile (which are identical with those measured at low water percentages); additionally, the ε_M values at high water percentages were confirmed from assays in extremely dilute solutions, through a special 9.50 cm optical length

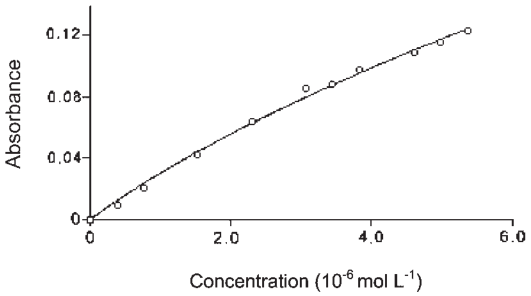


Figure 3. Experimental absorbance at 690 nm as a function of [Po] in 58% water in acetonitrile, 30.0 °C. The solid line was obtained from to fitting Eqn. (1)

cuvette. Figure 3 shows an example of the adjustment of the experimental points to the theoretical equation.

As shown in Fig. 3, the fitting is satisfactory, with a correlation coefficient (r) of 0.998, resulting in a value of 4.8 for log K_d. From each water–acetonitrile composition, the fittings of Abs_M against [Po] (series shown in Fig. 2) gave the K_d values presented in Table 1. Taking the Abs_M values at the Soret Band (430 nm), the same values of K_d are obtained.

The results given in Table 1 show a higher K_d as the water content in acetonitrile is increased, which means, as is predictable, that the aggregation process is favoured by the presence of water. For the other organic solvents mixtures, the plots of Abs_M as a function of [Po] exhibited the same negative deviation at high water contents (which starts from 55%, 30% and 50% of water for dioxane, methanol and ethanol, respectively; not shown). However, the model proposed by Eqn (1) (which assumes a dimer as the aggregate) does not work for these solvent series. This suggests that in these solvents systems, beyond monomers and dimers, the presence of higher aggregates (A) is possible. These results are in disagreement with those obtained in aqueous acetonitrile and DMSO solvents, where dimers are found as the aggregate dominant species.

To estimate the aggregation state, we postulate as a main equilibrium a single step between monomers and aggregates where dimers species are neglected, such as

$$x\text{M} \rightleftharpoons \text{A}$$

The equilibrium constant (K_a) can be written as

$$K_a = [\text{A}]/[\text{M}]^x$$

Table 1. Calculated K_d values for DiesterB in water–acetonitrile at 30.0 °C and the correlation coefficient (r)

Water(%)	Log K_d	r
55	4.5	0.999
58	4.8	0.998
60	5.1	0.991
62	5.4	0.990

where x is the aggregation number.
The total DiesterB concentration is given by

$$[\text{Po}] = [\text{M}] + x[\text{A}] \tag{2}$$

so

$$[\text{A}] = \frac{[\text{Po}] - [\text{M}]}{x}$$

Replacing and rearranging, one obtains

$$xK_a[\text{M}]^X = [\text{Po}] - [\text{M}]$$

Dividing by [M] and taking logarithms:

$$\ln[\text{M}] = \frac{\ln\{([\text{Po}]/[\text{M}]) - 1)\}}{\text{x} - 1} - \frac{\ln(x\,K_a)}{x - 1} \tag{3}$$

The plot of ln[M] versus ln{([Po]/[M])−1} gives the values of x and K_a from the slope (angular coefficient) and the intercept (linear coefficient), respectively. An example of this type of plot (for 33% of water in methanol) is shown in Fig. 4.

From Fig. 4, the slope $1/(x-1)$ resulted in 0.453, therefore $x = 3.2$. From the intercept, $-\{\ln(x\,K_a)\}/(x-1) = -13.06$, log K_a is calculated as 11.6. Table 2 summarizes the values of x and K_a calculated from each water composition in dioxane, methanol and ethanol obtained from the model described above. Additionally, the aggregation state for the water–acetonitrile system calculated by this method confirmed $x = 2$, i.e. dimer.

The data in Table 2 reinforce the observations in water–acetonitrile, where the high water content favours aggregation. Although the aggregation number for dioxane increases with water content, this values for methanol and ethanol are constant ($x = 3.2$). For these three cases, it is assumed that an average value of 3, i.e., the largest

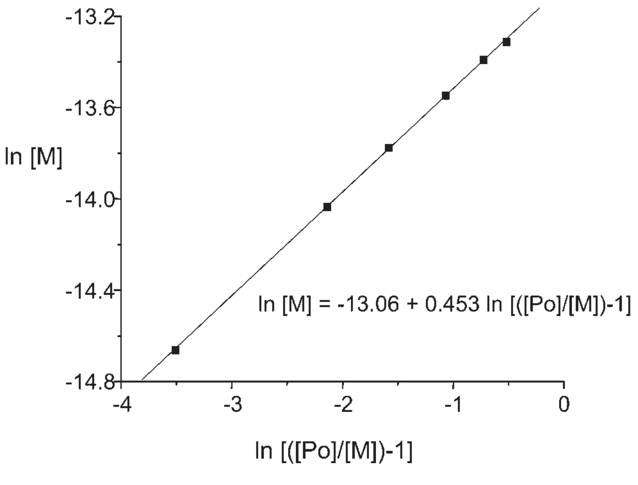


Figure 4. Plot of ln[M] vs ln{([Po]/[M])−1} for DiesterB for 33% water in methanol, 30.0 °C

Table 2. Values of aggregation number (x) and K_a for dioxane, methanol and ethanol in mixtures with water at 30.0 °C and the correlation coefficient (r)

Solvent	x	Water(%)	Log K_a	r
Dioxane	2.6	55	7.9	0.999
	2.9	58	10.2	0.983
	3.2	60	11.9	0.999
	3.6	65	14.9	0.995
Methanol	3.3	30	10.4	0.999
	3.3	32	11.1	0.999
	3.2	33	11.6	0.999
	3.2	35	12.1	0.966
Ethanol	3.1	50	9.6	0.993
	3.2	55	10.3	0.999
	3.2	58	10.8	0.999

aggregate in the equilibrium, is mainly a trimer; the presence of a quasi-isosbestic point leads to a reasonable assumption that the amount of dimer is not so significant and can be disregard in the K_a calculation.

Effect of the solvent dielectric constant

Similarly to the investigation of DiesterB in water–DMSO,[9] the dependence of the equilibrium constant on the solvent dielectric constant was analysed for all of the solvent systems. From the dependence of the equilibrium free energy (ΔG) on the dielectric constant (ξ), the equilibrium constant can be expressed as[15]

$$\log K = C - [W/(2.3RT)](1/\xi) \qquad (4)$$

where C and W are constants and ξ is the dielectric constant of the solvent (ξ_{mixt} for different mixtures), which can be roughly estimated by a linear relation as

$$\xi_{\text{mixt}} = \chi_{\text{organic solvent}}\xi_{\text{organic solvent}} + \xi_{\text{water}}\xi_{\text{water}} \qquad (5)$$

where χ is the molar fraction of each component and ξ represent the dielectric constant of the pure liquid. Taking the calculated equilibrium constants in Table 1 and from Eqn. (4), a plot of log K against $1/\xi_{\text{mixt}}$ furnishes the correlation of the aggregation process with the solvent parameter. A plot of such a correlation is given in Fig. 5 for the water–dioxane system.

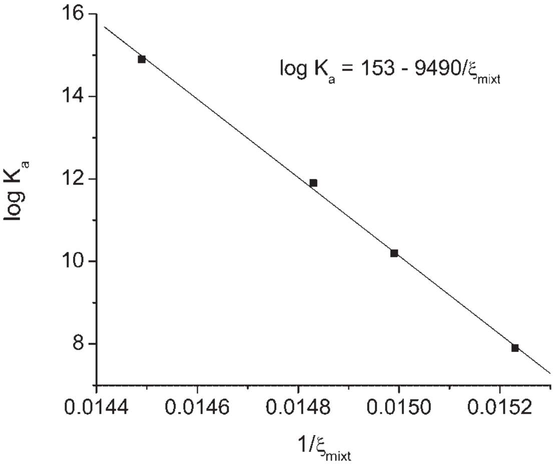


Figure 5. Log K_a versus $1/\xi_{\text{mixt}}$ for DiesterB in water–dioxane mixtures at 30.0 °C

As shown above, a linear dependence between log K_a and the average solvent dielectric constant is found, as would be expected for dipole–dipole interactions in solvents.[15] The same result was obtained for the other solvent mixtures (data from Table 2). From these plots, the pertinent linear equations (restricted to limited water contents) are presented in Table 3.

However, the correlations showed in Table 3 exhibit different equations for each solvent system. If the effect promoted by the solvent came uniquely from the dielectric constant, we would expect similar equations for monomer–dimer equilibria (water–DMSO and water–acetonitrile) and monomer–trimer (water–dioxane, water–methanol and water–ethanol) equilibria, which is not the case.

Effect of temperature

The temperature dependence on the aggregation equilibrium, fixing both the concentration of DiesterB and solvent composition, was investigated. Figure 6 shows the temperature effect on the equilibrium for the water–dioxane solvent system. As the temperature is raised, the aggregate is progressively consumed while the monomer concentration is increased, meaning that the aggregation process is an exothermic reaction ($\Delta H < 0$).

Table 3. Linear equations obtained for water in acetonitrile, dioxane, methanol and ethanol relating equilibrium constants and solvent dielectric constants for the mixtures (ξ_{mixt}) at 30 °C and the pure solvent dielectric constants (ξ)

Organic component	Equation	Validity range (water content, %)	ξ	r
DMSO[a]	Log $K_d = 25 - 1060/\xi_{\text{mixt}}$	30–60	46.6[a]	0.999
Acetonitrile	Log $K_d = 37 - 2190/\xi_{\text{mixt}}$	55–62	34.7	0.998
Methanol	Log $K_a = 50 - 2117/\xi_{\text{mixt}}$	30–35	32.7	0.993
Ethanol	Log $K_a = 38 - 1848/\xi_{\text{mixt}}$	50–58	24.3	0.995
Dioxane	Log $K_a = 153 - 9490/\xi_{\text{mixt}}$	55–65	2.2	0.999

[a] Data obtained at 25.0 °C.[9]

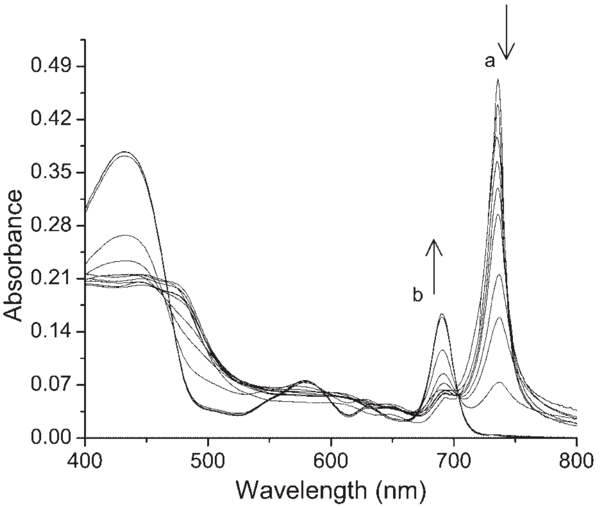


Figure 6. Temperature effect on the equilibrium (monomer and aggregate) in 60% water in dioxane. [DiesterB] = 5.34×10^{-6} mol l^{-1}; (a) 10.0 and (b) 60.0 °C. The arrows show the absorbance behaviour as the temperature is increased by 5.0 °C from (a) to (b)

As Fig. 6 shows, while the aggregate peak decreases, its maximum wavelength undergoes a small red shift, as the temperature is raised. For 60% water in dioxane the change is 730 nm (10.0 °C) to 735 nm (35.0 °C), for 50% water in methanol it is 725 nm (20.0 °C) to 743 nm (50.0 °C) and for 60% water in ethanol it is 732 nm (20.0 °C) to 736 nm (60.0 °C). This shift is not observed in water–acetonitrile, where its maximum wavelength at 740 nm is conserved. The aggregate maximum wavelength shift would be indicative that the aggregate state is modified by temperature (size and geometry). From the variation of the absorbance at 800 nm (baseline), it could not be excluded that some light scattering particles are present (e.g. colloidal higher aggregates).

However, considering that the aggregation number is unchangeable, and accepting the values from the fittings using Eqn. (1) ($x = 2$, Table 1) or Eqn. (3) ($x \approx 3$, Table 2), the equilibrium constants (K_d and K_a) are roughly evaluated for each temperature by $K = [A] / [M]^x$, taking [M] from the experimental absorbance at 690 nm and [A] from Eqn. (2). Taking the Gibbs–Helmholtz equation:

$$\ln K = -(\Delta H^\circ/R)[1/T] + \text{constant},$$

the logarithm of K as a function of $1/T$ should give a straight line, the slope of which should furnish the reaction enthalpy (ΔH) (Fig. 7).

However, as shown in Fig. 7 for DiesterB in 60% water in dioxane as an example, linearity was not observed, although ΔH can be evaluated as negative, i.e. the aggregation process is an exothermic reaction. The lack of linearity (found in the systems of water with acetonitrile, dioxane, methanol and ethanol) may be due to changes in the aggregate state with temperature or the rough K estimation or the formation of some higher aggregates promoting light scattering, or it can be an indicative that temperature affects each step in the multiple equilibrium (monomer–dimer–trimer) in a different way. Conclusive statements could be obtained from kinetic experiments, which we have been performing.

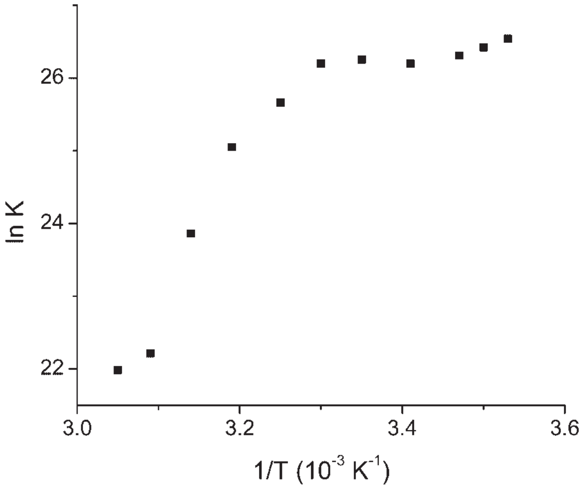


Figure 7. Relationship of the equilibrium constants (trimer) versus the reciprocal of temperature for DiesterB at 3.34×10^{-6} mol l^{-1} in 60% water in dioxane

CONCLUSION

The aggregation process of DiesterB in water–organic solvent systems is an exothermic reaction favoured by a high water percentage. For water–acetonitrile, the predominant aggregates are dimers, whereas for aqueous systems with dioxane, methanol and ethanol, the favoured final aggregates are trimers. In these cases, a small amount of dimer could be present, despite its being neglected in the K_a calculation). DiesterB behaves as a small dipole where the dependence of the equilibrium constants on the dielectric constant of the mixture permits the evaluation of K for any solvent composition in the range investigated (specific equations for each organic solvent). Moreover, despite the solvent polarity, the nature of the organic solvent plays an important role in the aggregation process, driving the aggregate size and the magnitude of the equilibrium constant.

Acknowledgements

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