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Direct blue dye solutions: photo properties

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Abstract

Weakly fluorescing azo dyes can be used as photothermal sensitizing agents in the photodynamic therapy (PDT) of some forms of cancer and other diseases. A desirable property of such a dye is that it will dissipate most of its excitation energy by nonradiative decay processes rather than by fluorescence or energy transfer to dioxygen. The maximum absorbance wavelengths, the fluorescence quantum yields Φ_F, the mean fluorescence wavelengths, and threshold dye concentrations below which Φ_F does not decrease with rising dye concentrations are reported for nine azo dyes that appear to have the correct optical properties for application as photothermal sensitizing agents. © 2000 Elsevier Science S.A. All rights reserved.

Keywords: Photodynamic therapy; Photothermal sensitizing dyes; Direct blue dyes; Photothermal spectroscopy; Fluorescence; Nonradiative quantum yields

1. Introduction

Photodynamic therapy (PDT) is a new type of treatment for tumors and certain diseases. This modality involves giving the patient a photosensitizing drug that accumulates in or is selectively retained by the diseased tissue. Subsequent illumination, typically with a laser, results in photo-damage or destruction of the diseased tissue with relatively little effect on the surrounding normal tissues. Tissue destruction can be mediated in several ways, including photochemical and photothermal processes. Photochemical processes include the generation of singlet oxygen and/or free radicals in oxygen-requiring reactions; these species, in turn, react with and destroy biomolecules in the diseased tissue [1,2]. In the photothermal mechanism, the sensitizer molecule is raised to temperatures sufficiently high to destroy closely associated biomolecules [3,4]. Several hundred types of photochemical photosensitizers have been studied for possible use in photodynamic therapy; however very little work has been done on possible photothermal sensitizers. An attractive feature of photothermal sensitizers is that they do not depend for their effectiveness on a continued local supply of dioxygen that rapidly disappears as the target tissue is damaged by other forms of photodynamic therapy. Properties of an effective photothermal sensitizer include (1) low toxicity to the organism in clinical doses, (2) tendency to concentrate in target biological tissues, (3) photoactivation by light of wavelengths (λ>600 nm) that penetrate biological tissues, (4) large extinction coefficients (ε) at the exciting wavelength (λ), (5) resistance to photodegradation, (6) a small quantum yield for intersystem crossing ($S_1 \rightarrow T_0$) and (7) a large nonradiative quantum yield (Φ_{nr}) compared to the fluorescence quantum yield (Φ_F).

Among the few examples of the biomedical application of photothermal sensitizers is the use of indocyanine green (a tricarbocyanine dye) with pulsed laser illumination for the successful treatment of rat mammary tumors [5]. Other cyanine dyes have also been suggested as possible photothermal sensitizers [6]. Neurons in mouse ganglia labeled with Procion blue HB (an amino anthraquinone derivative) show selective thermal damage on illumination with a pulsed laser [7]. Sudan black (a diazo dye) enhances the photothermal destruction of atheromatous plaque [8].

In the present study, several diazo dyes have been investigated as potential photothermal sensitizers for photodynamic therapy. The dyes investigated are pictured in Figs. 1 and 2 and Table 1 and include seven diazo, one triazo and one monoazo dye. Of these nine dyes, seven are classified as direct blue dyes, one as an acid black dye and one as a mordant blue dye. The classifications direct, acid and mordant refer to their manner of usage in the textile dyeing industry. In some instances, diazo dyes have been shown to localize in and be retained by tumors [9,10]. In the present study, fluorescence, triplet state and photobleaching char-

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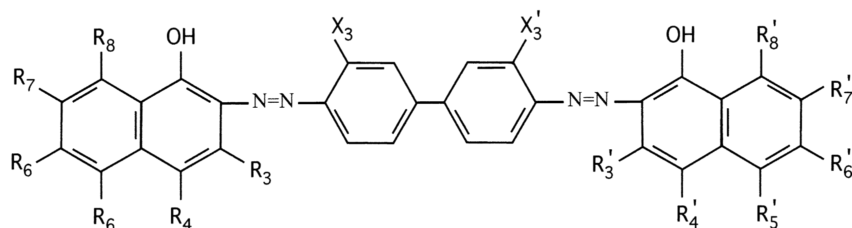

Fig. 1. Structures of the direct blue diazo dyes (see Table 1 for other details).

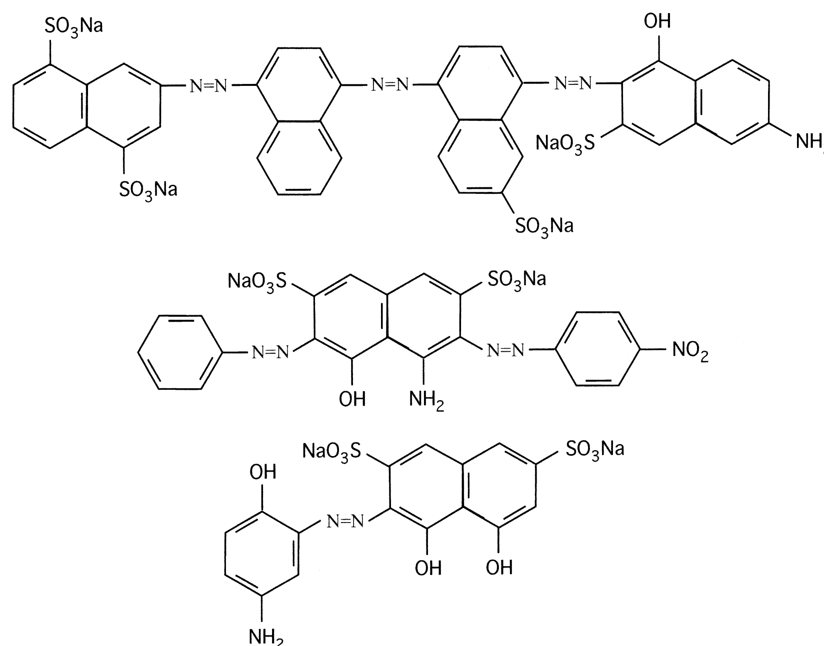

Fig. 2. Structures of additional azo dyes: Top, Direct Blue #71 (DB71); middle, Acid Black #1 (AB1); bottom, Mordant Blue #13 (MB13).

acteristics have been investigated using photothermal techniques and fluorescence and visible spectroscopies with the dyes in aqueous, aqueous micellar and methanolic solutions.

Several interactions are important in the solution chemistry of the investigated dyes. These include aggregation, tautomerism, and hydrogen bonding, as well as electrostatic, hydrophobic and acid/base interactions. The importance of each interaction is dependent on environment.

The behavior of the investigated polyazo dyes is likely to be similar to that of their monoazo counterparts, especially in those instances where the diazo dye is composed of two identical monoazo units with a plane of symmetry through the center of the molecule. Examples are DB1, DB6, DB14, DB15, DB25 and DB53. These direct blue diazo dyes are the primary focus of the present study.

The tautomerism of related monoazo compounds illustrated in Fig. 3 has been thoroughly explored [11–16]. Similar tautomerism must occur in the dyes investigated here. The azo-hydrazone tautomeric equilibrium occurs via an intramolecular hydrogen bond between the *o*-hydroxy group of the substituted azo naphthalene unit and the β-azo nitrogen. The intramolecularly hydrogen bonded six membered ring

Table 1
Direct blue diazo dyes

Dye name	R_3, R_3'	R_4, R_4'	R_5, R_5'	R_6, R_6'	R_7, R_7'	R_8, R_8'	X_3, X_3'
Direct Blue #1 (DB1)	H	H	SO_3Na	H	SO_3Na	NH_2	CH_3O
Direct Blue #6 (DB6)	SO_3Na	H	H	SO_3Na	H	NH_2	H
Direct Blue #14 (DB14)	SO_3Na	H	H	SO_3Na	H	NH_2	CH_3
Direct Blue #15 (DB15)	SO_3Na	H	H	SO_3Na	H	NH_2	CH_3O
Direct Blue #25 (DB25)	SO_3Na	H	H	SO_3Na	H	OH	CH_3
Direct Blue #53 (DB53)	H	H	SO_3Na	H	SO_3Na	NH_2	CH_3

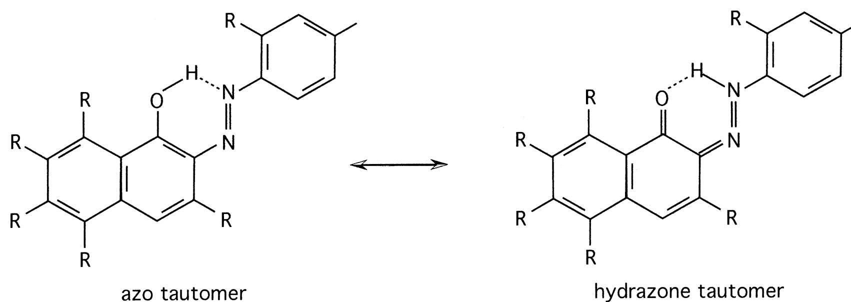

Fig. 3. Schematic representation of the azo-hydrazone tautomeric equilibrium of *o*-hydroxy azo dyes.

that is formed imparts a great deal of stability to the dyes. The energy of the intramolecular hydrogen bond formed in monoazo counterparts has been determined to have an energy of 5 to 10 kcal/mol [17]. For *o*-hydroxy azo compounds, as well as *o*-amino azo compounds, as in the case of AB1, the hydrazone tautomer has been determined to be the predominant species in solution [12–16,18–20]. The hydrazone tautomer becomes more stable as the hydroxy aromatic moiety increases in aromatic character, in other words, phenol<naphthol<anthrol [14]. Electron-withdrawing substituents such as sulfonate groups also favor formation of the hydrazone tautomer [16,21,22]. The hydrazone tautomer is more polar than the azo tautomer [20,22,23] and is consequently the predominant species in polar media.

The intramolecularly hydrogen bonded tautomeric structures orient the dye molecule into a planar structure eliminating the possibility of rotation about the naphthalene carbon-α-nitrogen bond. Although some of the polyazo dyes have been thought to be entirely planar, rotation about the central benzidine linkage is possible for the six diazo direct blue dyes investigated here. On the other hand, steric effects hindering rotation are possible in the five diazo direct blue dyes containing either an *o*-methyl phenyl or *o*-methoxy phenyl moiety as has been observed for DB15 [24]. Fluorescence of related *o*-hydroxy monoazo dyes as well as of some of the direct blue diazo dyes investigated here has been attributed to planarity induced by intramolecular hydrogen bonding [14,20,25–30].

Mordant Blue #13 (MB13), an *o,o′*-dihydroxy monoazo dye, is particularly subject to rigidification and planar orientation as a consequence of two adjacent intramolecular hydrogen bonding sites. It is not surprising then that MB13 has been observed to fluoresce strongly for an azo dye [31]. AB1 is also subject to intramolecular hydrogen bonding tautomeric interactions between the *o*-aminonaphthyl group and β-nitrogen of the corresponding azo linkage. Consequently, it would be expected to have a fairly rigid, planar structure. However, no indication of fluorescence for AB1 was found in the literature.

Intramolecular hydrogen bonding and steric factors favor the *trans*-configuration to such an extent, that the existence of any significant amount of *cis*-isomers is doubtful [32]. The *trans*-isomer has been illustrated in Fig. 3.

For the dyes investigated in the present study, the sulfonate groups are of particular interest. Although the sulfonate groups are primarily utilized to solubilize the dyes in aqueous media, they affect other aspects of dye behavior, most notably aggregation. At the essentially zero ionic strength conditions prevailing in the present study the four negatively charged sulfonate groups on each monomer act against aggregation of the dye molecules. Furthermore, the dyes investigated here are not expected to be aggregated at the concentrations studied (in general between 10^{-8} and 10^{-6} mol dm^{-3}) [32,33]. In fact, DB1 has been determined by UV/VIS spectral measurements to exist in monomeric form in such a concentration range [34,35].

2. Materials and methods

Mordant Blue #13 (MB13), commonly referred to as Plasmocorinth B, Direct Blue #53 (DB53), commonly referred to as Evans Blue, and Direct Blue #71 (DB71) were obtained from Aldrich. Acid Black #1 (AB1) and Direct Blue #14 (DB14), commonly referred to as Trypan Blue, were obtained from Kodak. Direct Blue #1 (DB1), Direct Blue #25 (DB25) and Direct Blue #6 (DB6) were obtained from Pfaltz and Bauer. The manufacturer of Direct Blue #15 (DB15) could not be identified.

Polyazo dyes are difficult to purify or to obtain in high purity from a manufacturer. Chromatographic separation other than by thin layer chromatography (TLC) is problematic. Consequently, the received dyes were recrystallized from ethanol or 50/50 methanol/water. The purity of the dyes was checked by TLC using a solvent composed of *n*-butanol/pyridine/water (1:5:4 by volume) [36] and a stationary phase of silica gel on aluminum or chromatography paper (3MM Chr), both obtained from Whatman. The chromatography paper was found to be most effective. In all cases, with the exception of DB14 and AB1, significant purification of the received dyes was achieved upon repeated recrystallization. DB14 and AB1 were utilized ‘as is’ after four successive recrystallizations.

Rhodamine 640 perchlorate and cresyl violet were obtained from Exciton and used as received. Sodium dodecyl sulfate and cetyltrimethylammonium bromide from Sigma,

sodium hydroxide (analytical grade) and hydrochloric acid (analytical grade) from Mallinckrodt, methanol (Omni-Solv, spectrophotometric grade), carbon tetrachloride (Omni-Solv, spectrophotometric grade) and pyridine from EM Science, acetone (PHOTREX-spectrophotometric grade), ethyl ether (anhydrous) and glacial acetic acid from JT Baker, Triton X-100, *N*,*N*-dimethylformamide (HPLC grade), methyl sulfoxide (HPLC grade) and diethylene glycol from Aldrich and ethanol (punctilious) from Quantum were all used as received. Ultrapure water was used from a Corning MP-190 water purification system connected to a MP-1 water still. In all sample solutions dye concentrations were in the range between 10^{-8} and 10^{-6} mol dm^{-3} which, with no added electrolyte present, corresponded to essentially zero ionic strength conditions.

A photothermal beam deflection experiment [37] was used to establish that no triplet states are detectable in these dye molecules. The equipment and methodology have been described elsewhere [38]. The sample cell was made of quartz with a 1 cm optical path length. Observed signals were checked for linearity with respect to energy for all concentrations of the dyes used. Solutions were deaerated to approximately 3×10^{-6} Torr. The signature of a triplet state in a photothermal beam deflection experiment is a comparatively slow release of heat from the excited dye molecule.

Fluorescence measurements were made on a Perkin–Elmer MPF-66 Fluorescence Spectrophotometer. Emission spectra were corrected using a totally reflecting surface supplied by the manufacturer. A Perkin–Elmer Lambda 9 spectrophotometer was used for absorbance measurements. Refractive indices (25°C) were taken to be 1.3288 and 1.3326 for methanol and water, respectively [39]. Determination of the refractive indices for Triton-X, cetyltrimethylammonium bromide and sodium dodecyl sulfate has been described elsewhere [40]. The refractive indices were taken to be 1.3360±0.0004, 1.3336±0.0005 and 1.3355±0.0006, respectively. All spectral measurements were made at an ambient temperature of 24.0±0.2°C.

Relative fluorescence quantum yields Φ_F of the investigated dyes were determined relative to cresyl violet in methanol from fluorescence measurements according to the following relationship [41–44]:

$$\phi_F = \phi_{F_{ref}} \left(\frac{A_{ref}}{A}\right)\left(\frac{n_D}{n_{D_{ref}}}\right)\left(\frac{a}{a_{ref}}\right) \quad (1)$$

where A is the fraction of light absorbed, n_D is the index of refraction of the solvent, a is the area under the fluorescence peak and the subscript ref denotes a reference compound. This expression is only valid, however, assuming that the relationships between absorbance and concentration and the area under the fluorescence peak and concentration are linear. At concentrations of cresyl violet above approximately 10^{-6} M the relationship between the area under the fluorescence peak and concentration is no longer linear. To compensate for this nonlinearity, solutions of matching absorbances of cresyl violet and the investigated dyes in the solvent systems studied were used to determine Φ_F from the above equation. A value of the fluorescence quantum yield for cresyl violet in methanol of $\Phi_{F_{ref}}=0.67$ was used for cresyl violet solutions of 6×10^{-7} M or less [38]. For more concentrated solutions values of $\Phi_{F_{ref}}$ were taken from Table 1 of reference [38].

Photobleaching measurements were made on the dyes using illumination provided by a 500 W quartz halogen lamp system with interference filters corresponding closely to the absorbance maximum of the dye being investigated. This resulted in incident power densities of 3 to 10 mW cm^{-2}. A 2.5 ml aliquot of the sample dye solution was placed in an 1 cm optical path length quartz cuvette and the absorbance spectrum recorded. The dye was then illuminated in air with stirring. The cuvette was removed from illumination and the absorbance spectrum recorded at regular time intervals. Changes in absorbance maxima as well as the appearance of any new absorbance peaks as a result of illumination were monitored with respect to time. The quantum yields of photobleaching were then calculated as: (initial rate of disappearance of dye molecules)/(initial rate of absorption of photons). The error associated with such measurements is approximately 10%. Similar measurements were made using pulsed excitation of λ=610 nm from a Lambda Physik FL2002 dye laser pumped by a frequency doubled Quanta Ray DCR-2 Nd:YAG laser. Further details of the method may be found elsewhere [45–47].

3. Results and discussion

3.1. Color changes with changing pH

The dyes investigated in this study with between four to eight protolytic sites experience acid/base equilibria. In neutral aqueous media only the sulfonate groups are ionized and charges range from −2 for MB13 to −4 for the six structurally similar direct blue dyes. While many azo dyes have been used as acid/base indicators, the intramolecular hydrogen bond of the dyes investigated in this study impart considerable chemical stability, and no color changes are observed over a wide pH range. Removal of the phenolic proton in hydroxy azo dyes, however, always produces a color change which in the presence of an intramolecularly hydrogen bonded *o*-hydroxy azo dye occurs at pH>11 [32,48,49]. Thus, DB53 undergoes a series of color changes starting at a pH of approximately 11.2.

3.2. Photobleaching, photothermal and absorbance data

Dye bleaching, with quantum yields above the detection limit of approximately 1×10^{-6} or the appearance of new absorbance peaks was not observed for any dye in the photobleaching measurements. This was true for the aqueous solutions, aqueous Triton X-100 (TX) solutions, and methanolic solutions. The observed stability of these dyes is consistent

Table 2
Maximum absorbance wavelength (λ_{max}), fluorescence quantum yield (Φ_F), mean fluorescence wavelength (mfw) and low concentration limit[a] (LCL) for several azo dyes

Dye	Methanol			Water			Aqueous TX			LCL (M)
	λ_{max} (nm)	Φ_F ($\times 10^{-3}$)	mfw (nm)	λ_{max} (nm)	Φ_F ($\times 10^{-5}$)	mfw (nm)	λ_{max} (nm)	Φ_F ($\times 10^{-3}$)	mfw (nm)	
MB13	528	6.2±1.8	628±4	530	11±0.5	620±2	538	0.63±0.05	625±1	1×10^{-5}
AB1	581	NDF[b]	–	589	NDF[b]	–	627	0.016±0.001	643±3	2×10^{-4}
DB71	584	<0.0005	656±1	589	NDF[b]	–	601	0.0095±0.0007	643±3	3×10^{-6}
DB6	594	4.0±0.3	630±1	587	8.1±0.8	635±1	606	4.9±0.7	631±1	1×10^{-4}
DB25	598	2.6±0.2	633±3	590	7.5±0.6	636±3	614	5.6±0.6	635±3	4×10^{-6}
DB14	605	4.3±0.4	638±1	596	7.9±0.8	623±1	588	10±1	637±2	1×10^{-6}
DB53	614	3.9±0.3	638±2	608	6.1±0.6	638±3	630	3.8±0.2	642±2	1×10^{-6}
DB1	621	2.4±0.2	640±1	620	2.2±0.2	641±1	635	3.0±0.2	642±3	1×10^{-6}
DB15	622	2.2±0.2	642±1	620	1.5±0.1	640±2	635	2.3±0.2	644±2	3×10^{-6}

[a] The low concentration limit (LCL) is that dye concentration below which Φ_F does not decrease with increasing concentration.
[b] No detectable fluorescence.

with literature reports in which the significant stability of similar *o*-hydroxy azo dyes has been attributed to the formation of an intramolecular hydrogen bond [17,32,50]. Other factors that contribute to the photostability of azo dyes are a large number of sulfonate groups, dye aggregation and an increasing number of azo groups such that stability increases in the order of monoazo<diazo<triazo, etc. [32]. Several dyes in this study have also been found to be quite thermally stable [32]. The photochemical and thermal stability of these dyes are ideal for their use as photothermal sensitizers in photodynamic therapy.

Photothermal beam deflection investigations of the dyes indicated the absence of triplet states in both aerated and deaerated solutions. MB13 was not investigated due to a lack of significant absorbance at λ=610 nm. The absence of a detectable triplet state is not surprising for such large organic dyes where many modes of nonradiative relaxation and charge transfer (CT) exist [14,16,51–53].

Visible absorbance and fluorescence data for the dyes investigated in this study are listed in Table 2. All of the dyes fluoresce slightly and have large molar absorptivities, ε, which are on the order of 10^4 to 10^5 dm^3 mol^{-1} cm^{-1}. There are two notable exceptions with respect to ε, DB6 and AB1. The value of ε for DB6 is approximately 1×10^3 dm^3 mol^{-1} cm^{-1}. The lower absorptivity for a dye such as DB6 has been attributed to the lack of an alkyl or alkoxy substituent on the benzidine moiety in contrast to the other five structurally similar direct blue diazo dyes [54]. The value of ε for AB1 is also approximately 1×10^3 dm^3 mol^{-1} cm^{-1}. The reason for the lower absorptivity of AB1 is unknown.

Visible absorbance spectra of the dyes consist of two overlapping peaks in aqueous, aqueous TX and methanolic solutions. This is indicated for DB53 in Fig. 4. The main peak occurs at a wavelength longer than the minor peak, the minor peak appearing as a blue shifted shoulder on the main absorbance peak. The spectra reflect the azo-hydrazone tautomeric equilibrium in solution where the long wavelength absorbance maximum has been assigned to the hydrazone tautomer [23,33,55,56]. The spectra are thus consistent with the hydrazone being the predominant tautomeric species in solution for the dyes investigated. The only exception is DB71 for which the azo form of the dye appears to be the predominant species in solution. This is plausible, however, since steric effects tend to favor the azo tautomeric form of DB71.

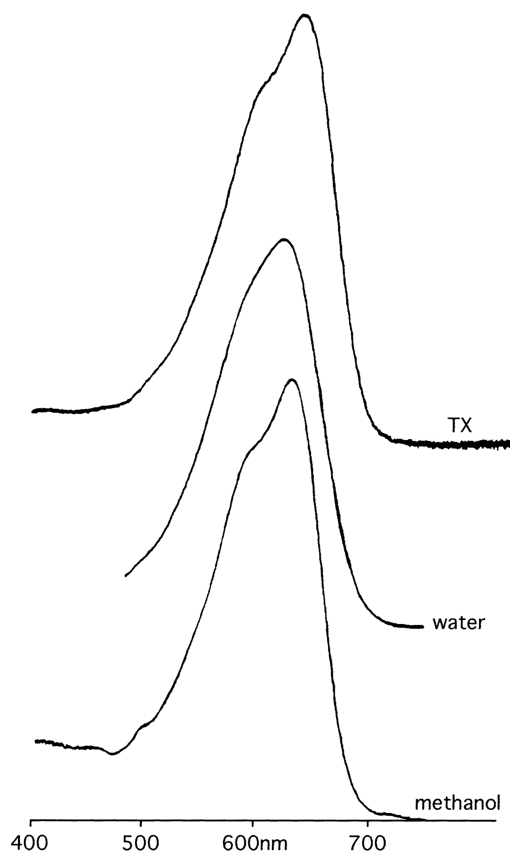

Fig. 4. Visible absorbance spectra of DB53(Evans Blue) in aqueous TX (nonionic detergent), aqueous, and methanolic solutions.

The ratio of hydrazone to azo tautomer appears constant from dye to dye as well as in both methanolic and aqueous TX solutions. In all cases an increase in the amount of azo tautomer is indicated in aqueous solutions perhaps because of the greater capacity of water to form strong intermolecular hydrogen bonds that favor formation of the azo tautomer. The exception to the above behavior is DB14, for which the azo tautomer was observed to increase in concentration in going from aqueous to the aqueous TX micellar solutions. The reason for this exceptional behavior is unknown.

The visible spectra of azo dyes are notoriously insensitive to changes in solvent, aggregation, etc. Consistent with this fact, no visually detectable changes were observed in any of the dye spectra in a particular solvent, including water at neutral pH, over a wide concentration range. Thus, it was impossible to ascertain aggregate formation from the spectral data in hand. Changes in the spectra, however, were observed in acidic and/or basic media. Thus, while no or very small changes in the shape of absorbance spectra of DB53 were observed in a wide variety of solvents, significant changes were observed in acidic and basic aqueous solutions. In acidic solutions, ε decreased dramatically, the short wavelength azo shoulder disappeared and a new shoulder (peak) appeared to the red of the hydrazone peak. The origin of the new shoulder (peak) is unclear. In basic solutions, the hydrazone peak vanished and only one peak, that of the azo form, was still present and slightly blue shifted. The effect could be seen with the naked eye as the solution changed color from blue to violet around pH 11. This change corresponds to deprotonation of the *o*-hydroxy hydrogen which restricts the dye to the azo configuration.

Although Beer's law was obeyed over a wide concentration range for all the dyes in Table 2, this does not assure the absence of aggregate formation since adherence to Beer's law can be expected for a constant particle size (i.e., a constant degree of aggregation) [57]. Aggregation can generally be eliminated in micellar solutions. Although the spectra in aqueous micellar and methanolic solutions appear nearly identical, it should be noted that the difference observed in the aqueous spectra assigned to an increase in the azo tautomer may instead reflect the presence of aggregates. A study [13] has found that the azo-hydrazone tautomerism of an *o,o'*-dihydroxy azo dye is affected by the state of dye aggregation which in turn is affected by the solvent. The authors concluded that hydrazone tautomers were the predominant species in the aggregated state. Evidence for aggregate formation based on the absorbance data at hand is inconclusive.

3.3. Fluorescence data

The fluorescence data reported in Table 2 are for the 'low concentration limit', the concentration below which Φ_F was not observed to decrease with increasing concentration of dye. The 'low concentration limit' has been indicated for each dye in Table 2. A decrease in Φ_F and an increase in the value for the mean fluorescence wavelength (mfw) were observed for all the dye solutions for which fluorescence was detectable with increases in concentration above the 'low concentration limit'. The decrease in fluorescence with increasing concentration may be due, at least in part, to one of the following three phenomena: the formation of a sandwich (or stacked) dimer or higher aggregate, inner filter effects [58–64], or concentration quenching [65–67].

The fact that the investigated polyazo dyes fluoresce slightly is significant for two reasons. First, azo dyes, as a general rule, do not fluoresce. Where fluorescence has been observed in monoazo counterparts of the investigated diazo dyes, it has been attributed to the rigidity imparted by the intramolecular hydrogen bond formed as a consequence of the *o*-hydroxy azo structure. It can be inferred that the same effect is responsible for the fluorescence observed from the dyes investigated in this study. Secondly, while some of the direct blue diazo dyes studied here have been used as fluorescent tracers, they have generally not been observed to fluoresce when unbound (to proteins) in solution [25–27,29,30]. The results, however, clearly indicate detectable fluorescence from unbound dyes in both methanolic and aqueous solutions. While the dyes fluoresce, the Φ_F values are low, and the level of fluorescence is not great enough to interfere with their effectiveness as photothermal sensitizers. Even when bound, as in the case of aqueous TX solutions, the Φ_F values are of the order of only 10^{-3}.

Although there are two tautomeric species, the hydrazone tautomer has been determined to be the fluorescent species in solution [14,20,28]. Emission spectra of similar monoazo dyes have been determined to mirror approximately the hydrazone absorption peaks [20]. The fact that only one species is responsible for fluorescence can be seen by looking at the fluroescence spectra. The fluorescence spectra of the polyazo dyes investigated in this study were not found to be mirror images of the corresponding absorbance spectra. This nonmirror image relationship between the absorbance and fluorescence spectra has also been observed for DB14 and monoazo counterparts [14,26]. Representative fluorescence spectra for DB53 (Fig. 5) indicate one fairly symmetrical fluorescence peak suggesting a single molecular species as opposed to the corresponding absorbance spectra in Fig. 4 which clearly indicate the presence of at least two molecular species. This was observed in aqueous, aqueous TX and methanolic solutions. The fact that the hydrazone tautomer is responsible for the observed fluorescence is reasonable since upon excitation the azo nitrogens of the dyes will become more basic and the hydroxide group more acidic [53,68,60]. Changes in pK_a values of 5 to 10 units upon excitation are common [69–72]. Consequently, the hydrazone tautomer (with the hydrogen bonded to the β-nitrogen of the azo group and intramolecularly hydrogen bonded to the *o*-oxazone) will be the favored species in the excited state. Thus, upon excitation, a charge transfer process favoring the formation of the hydrazone tautomer probably takes place. The species that is the result of such a charge transfer, in this case the hydrazone tautomer, is the species responsible

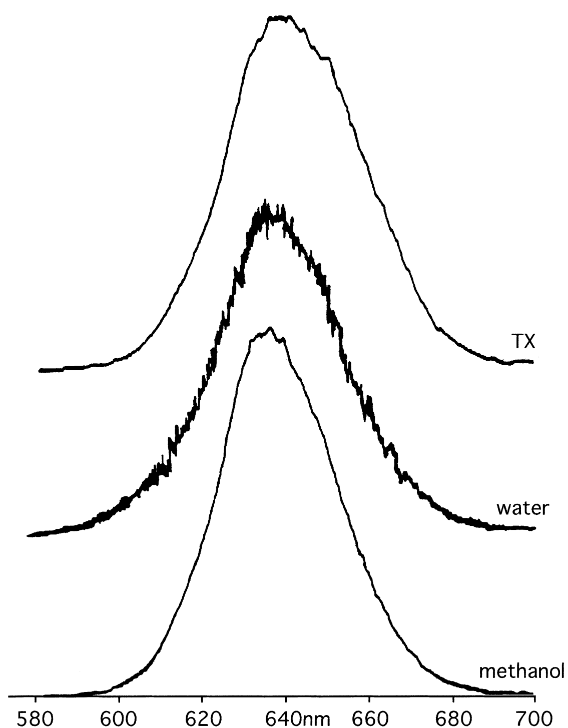

Fig. 5. Fluorescence spectra of DB53 (Evans Blue) in aqueous TX (nonionic detergent), aqueous, and methanolic solutions.

for any observed fluorescence [53]. The fluorescence of the hydrazone tautomer, in addition to being attributed to an induced planar orientation, has also been attributed to deconjugation of the azo group with the rest of the dye molecule [27]. The large Stokes shifts observed for many of the investigated dyes is also indicative of a charge transfer process [53]. Especially noteworthy is MB13 for which the Stokes shift is of the order of 100 nm.

This charge transfer process, however, is also probably responsible for quenching the fluorescence of the hydrazone tautomer upon relaxation and subsequent equilibration with the azo tautomer. Another significant channel for nonradiative relaxation of the investigated dyes is through vibrational interaction of the intermolecular hydrogen bonds with the solvent molecules [52,73,74]. Such quenching or nonradiative effects can be expected to be enhanced in water where strong hydrogen bonding interactions occur. Additional nonradiative modes of deactivation in aqueous solution include high frequency –OH vibrations [75] and aggregate formation.

With the exception of AB1 and DB71, the values for Φ_F and the Stokes shift of the dyes decrease with an increase in the value of the wavelength of maximum absorbance in methanolic and aqueous solutions. This may reflect differences in ground state hydrazone populations, in the strength of the intramolecular bond or in the degree of aggregation. The decrease also probably reflects the fact that the probability of nonradiative vibronic coupling between the ground and excited state increases as energy differences between the ground and excited state decrease [75–77].

The very low Φ_F values for DB71 are consistent with steric factors that inhibit a planar orientation and favor a degree of rotation about the azo linkages. The lack of fluorescence from AB1 is a little more difficult to understand. AB1 has both an amino and a hydroxy group situated ortho to the azo linkage. Although a tautomeric equilibrium can be expected to exist for the hydroxy group, the orientation with respect to the amine group can be expected to consist only of the azo form as already mentioned. While AB1 may have a significant hydrazone tautomer population upon excitation, rotational deactivation processes at the other azo linkage could provide an important channel for nonradiative relaxation. Additional modes of nonradiative relaxation arise from the high aggregating potential of AB1 caused by the presence of the p-NO_2 group [27,33,78,79] and quenching impurities that may be present because of difficulties in purifying AB1. Such impurities may also be present in the case of DB14, where a similar problem was encountered. However, in the case of DB14, excitation at λ=610 nm has been determined to excite selectively DB14 as opposed to any common impurities found in the dye [26]. Considering that the fluorescence observed from DB14 is of the same order of magnitude as that from DB53, a structural isomer, it is likely that no significant quenching of the fluorescence due to impurities occurred. In fact, the values of Φ_F reported for DB14 and DB53 in Table 2 are quite similar. The agreement in Φ_F values for the structural isomers DB1 and DB15 is excellent.

3.4. Micellar data

Absorbance and fluorescence characteristics of the dyes under investigation were studied in anionic sodium dodecyl sulfate (SDS) micellar, cationic cetyltrimethylammonium bromide (CTAB) micellar and nonionic TX micellar solutions. No changes in absorbance or fluorescence characteristics were observed in the SDS micellar solutions presumably because of strong electrostatic repulsion between the anionic dye and the anionic sufactant head group. Only a slight red shift in absorbance as well as a slight increase in fluorescence (∼5%) was observed for the dyes in CTAB solutions. Although electrostatic interactions between an anionic dye and a cationic sufactant head group are favorable in the case of CTAB, other factors are apparently unfavorable for interaction such as the small interior dimensions of the CTAB micelle. Finally, significant red shifts in absorbance as well as large increases in fluorescence (on the order of two orders of magnitude) were observed in the nonionic micellar TX solutions. Thus, a discussion of the nature of the interactions between the investigated dyes and the TX micelles is clearly warranted.

The increase in fluorescence as well as the red shift in absorbance for the dyes in going from aqueous to aqueous TX solutions (see Table 2) indicates a strong dye/micelle in-

teraction [80–83]. The increase in fluorescence of the dyes upon interaction with the TX micelles may result from several factors. First, dye interaction with the micellar environment may diminish nonradiative deactivating interactions with water. In other words, the microenvironment offered by the TX micelle may stabilize the excited state of the dye and protect it from nonradiative deactivation processes [81]. Second, aggregating processes may be eliminated or reduced by the more hydrophobic environment provided by the TX micelles. Finally, interaction of a dye with the TX micelle may result in a rigidification of the dye structure primarily through restricted rotational modes of deactivation, decreasing the number of channels available for nonradiative relaxation.

Electrostatic interactions of these azo dyes with TX micelles is not expected due to the nonionic nature of the TX micelles. It is also unlikely that the dyes, because of their polar hydroxyl and amine groups and their charged sulfonate groups will localize in the significantly hydrophobic interior of the TX micelles. The interaction between some monoazo dyes and a series of nonionic surfactants has been shown to be hydrophobic in nature, occurring between the dyes and the polyoxyethylene (POE) chains of the surfactant [84]. The POE chains of TX are exposed to water [85,86] so that the dyes are essentially placed in a hydrophobic environment that is exposed to water [87]. Some degree of van der Waals ion–dipole or dipole–dipole interactions may also be expected between the dyes and the polar POE chains [79,87,88].

Although the interactions of the investigated dyes with proteins and the dyes with TX micelles differ, it seems unlikely that the observed fluorescence will be significantly larger when the dyes are bound to proteins than when bound to TX micelles. Even if the Φ_F values were an order of magnitude larger when bound to proteins than when bound to TX micelles, the dyes would still be good candidates for photothermal sensitizers because of their large nonradiative quantum yields.

4. Conclusion

Many of the chemical and photophysical properties of the diazo dyes in this study are similar to those of their monoazo counterparts. Most of the dyes investigated were observed to fluoresce slightly, even in nonmicellar solutions. Relative fluorescence quantum yields for these dyes were determined in methanolic, aqueous and aqueous TX micellar solutions for the first time. All the polyazo dyes investigated appear to be good candidates for photothermal sensitizers in photodynamic therapy.

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