Journal of Fluorescence, Vol. 7, No. 4, 1997

Fluorescence Reduction by a Triplet-State Population

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Received October 27, 1997; revised December 24, 1997; accepted December 30, 1997

The range of applications for fluorescent dyes in medicine and biology is increasing greatly. At the same time, the demands on the dyes are getting bigger. The dye molecules are often expected to emit maximum-intensity fluorescence. The primary requirement for this is naturally a high fluorescence quantum yield. Beyond this, it must be considered that fluorescent molecules located in a meta-stable triplet state are not able to emit fluorescent radiation. This represents a reduction of the intensity of the fluorescence, which is generally underestimated in its importance. The size of this perturbation can be grasped by knowledge of the quantum yield for occupation of the triplet state and its lifetime. Unfortunately these parameters are completely unknown, even for frequently used dyes. On investigating some medically and biologically relevant dyes, it is shown that the attainable fluorescence intensity could be reduced strongly by occupation of the triplet state.

KEY WORDS: Advanced transient spectrometer; time-resolved spectroscopy; fluorescence reduction; triplet state; biologically relevant dyes.

INTRODUCTION

The application of fluorescent dyes has a wide spectrum. Beside the classical fields such as dye lasers and mode locking,[1–4] areas in medicine[5] and engineering are profiting from the properties of these dyes. Especially, the fact that fluorescence can be detected easily and is extremely sensitive makes them the first choice in ultraanalytical problems down to the single-molecule level.[6] For most applications the primary requirement is a high fluorescence quantum yield. It must, however, be considered that molecules located in a meta-stable triplet state are not able to emit fluorescent radiation. This represents a reduction of the averaged intensity of the fluorescence.

For most dyes the fluorescence quantum yield is measured and its solvent dependency is investigated.[7,8] From these measurements empirical models, to predict the fluorescence quantum yield, are developed. These models can be used only very poorly to predict the properties of the triplet state. This may be caused by the scarce and controversial information published about this state.[9] Therefore the importance of the fluorescence reduction, even for frequently used dyes, is unknown.

In Ref. 9 a technique is described which allows highly precise detection of transient absorption in the microsecond region. With this technique the physical properties of excited states of some fluorescent dyes have been investigated.[10] In the present paper this technique is used to measure the solvent dependency of the triplet-state properties of four medically and biologically relevant dyes.[11–19] The results are interpreted with regard to their importance for the discussed applications.

THEORY

In order to calculate the fluorescence intensity I_{fl} emitted by a dye solution, it is useful to determine the concentration c_1 of the emitting state (first excited singlet

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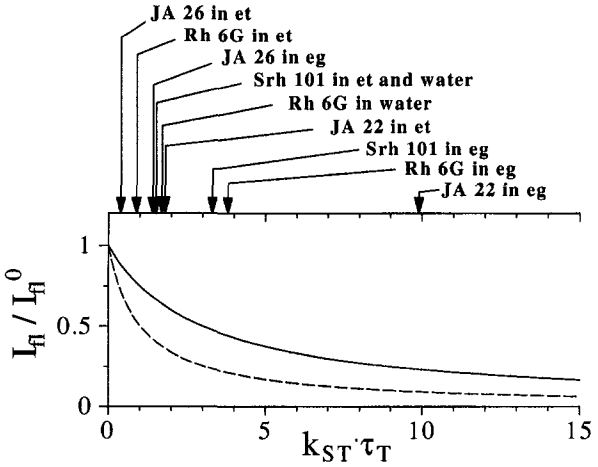

Fig. 1. Influence of $k_{ST} \cdot \tau_T$ on the intensity of fluorescence. (——) Theoretically calculated curve [Eq. (5)]; excitation power, 10 mW (He–Ne laser). (---) Theoretically calculated curve [Eq. (5)]; excitation power, 1 W (Ar-ion laser). $F = 4 \cdot 10^{-12}$ m^2; $\varepsilon = 10^5$ L/(mol·cm). Srh 101, sulforhodamine 101; Rh 6G, rhodamine 6G; eg, ethylene glycol; et, ethanol. Arrows are measured values of $k_{ST} \cdot \tau_T$.

state). For further considerations, only the relation

$$I_{fl} \propto c_1 \tag{1}$$

is important. In the case of a continuous irradiated dye solution the steady-state rate equation of the first excited singlet state (S_1) and the lowest triplet state (T_1) is given by

$$\frac{dc_1}{dt} = 0 = \frac{I_{ex} F \varepsilon_0 c_0 d \ln 10}{N_A V} - \frac{1}{\tau_1} c_1 \tag{2a}$$

and

$$\frac{dc_T}{dt} = 0 = c_1 k_{ST} - c_T \frac{1}{\tau_T} \tag{2b}$$

where

c_0 = concentration of molecules in the ground state
c_1 = concentration of molecules in the S_1 state
c_T = concentration of molecules in the T_1 state
ε_0 = molar decadic extinction coefficient of the ground state at the excitation wavelength
τ_1 = decay time of the S_1 state
k_{ST} = rate for intersystem crossing
I_{ex} = number of excitation photons per s and cm^2
F = cross section of excitation focus
d = path length (perpendicular to excitation direction)
V = excited volume
N_A = Avogadro constant

Equation (2) is valid for $\varepsilon_0 \cdot c_0 \cdot d << 1$ and absence of excited-state absorption.

With a constant overall dye concentration c,

$$c = c_0 + c_1 + c_T$$

one obtains from Eq. (2)

$$c_1 = \frac{c\,(\tau_1/\Delta t)}{1 + (\tau_1/\Delta t)\,(1 + k_{ST}\,\tau_T)} \tag{3a}$$

where

$$\Delta t = \frac{N_A F h c}{P \lambda \varepsilon_0 \ln 10} \tag{3b}$$

and

h = Planck constant
P = excitation power
λ = excitation wavelength

The variable Δt represents the averaged time interval between two absorption processes of one dye molecule in the ground state. Equation (3) shows that the population of the nonemitting triplet state (represented by the product $k_{ST} \cdot \tau_T$) leads to a reduction of the concentration c_1. Owing to Eq. (1) this results in a reduction of the fluorescence intensity. The influence becomes relevant if Δt is of the order of τ_1 and increases with the density of excitation photons and therefore with the excitation power.

In the case of prohibited intersystem crossing ($k_{ST} \cdot \tau_T = 0$), the dye solution emits a fluorescence intensity I_1^0 and the population c_1^0 of the S_1 state is then given by Eq. (3) as

$$c_1^0 = \frac{c\,(\tau_1/\Delta t)}{1 + (\tau_1/\Delta t)} \tag{4}$$

With Eqs. (1), (3), and (4), one obtains for the relative decrease in the fluorescence intensity

$$\frac{I_{fl}}{I_{fl}^0} = \frac{1 + (\tau_1/\Delta t)}{1 + (\tau_1/\Delta t)\,(1 + k_{ST}\,\tau_T)} \tag{5}$$

Equation (5) can be discussed in the following way. In the case of prohibited intersystem crossing, each dye molecule is able to emit photons in time intervals of its fluorescence lifetime τ_1. After conversion into the triplet state, on the contrary, fluorescence emission is prevented during the triplet lifetime. In this case, on average a dye molecule is able to emit photons in time intervals given by $\tau_1(1 + k_{ST}\tau_T)$. Figure 1 shows the relative reduction of the fluorescence I_{fl}/I_{fl}^0 calculated by Eq. (5). Obviously this process cannot be neglected even for an excitation

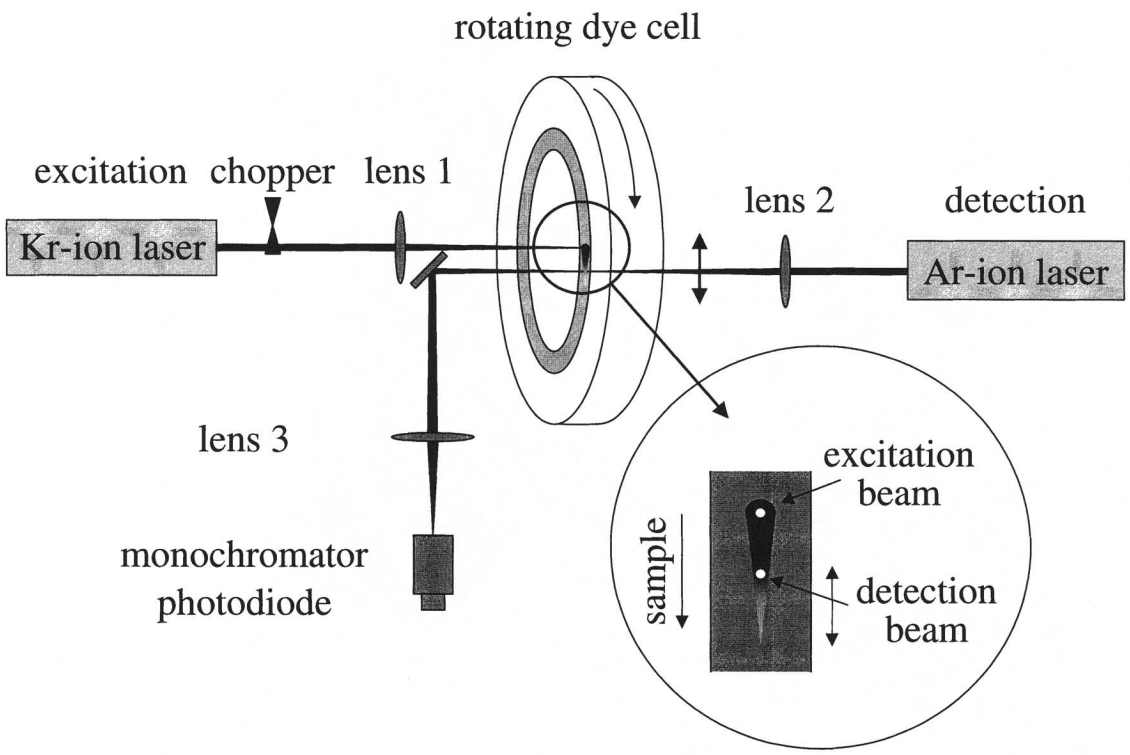

Fig. 2. Kr-ion laser, Type CR-3000K (Coherent Radiation); Ar-ion laser, Type Innova 70 (Coherent Radiation); pockels cell, Type 3031-FW (Fastpulse Technology), driven by a frequency generator (Type PM 5134; Philips); rotating dye cell, specially made, fused silica (Hellma); monochromator, Type H20 (Jobin Yvon); photodiode, Type 404 (Spectra Physics); lens 1, achromatic lens, focal length = 60 mm; lens 2, achromatic lens, focal length = 60 mm; lens 3, focal length = 100 mm.

intensity appearing in a focused low-power He–Ne laser beam.

EXPERIMENTAL

In order to measure the key parameters k_{ST} and τ_T [see Eq. (5)] we use an experimental technique which is described in detail in Refs. 9 and 10. Briefly described, an excitation beam is focused into the fast-flowing sample and produces a characteristic distribution of transient states. A probe beam, also focused into the track, crosses the sample at a selected position downstream (see inset in Fig. 2). The vertical adjustment of the probe beam is achieved by moving the focusing lens with a stepper motor (Type LM 60, Owis), which is controlled by a computer. By comparison of the probe transmission X_1, having transient states populated (excitation beam on), with the transmission X, having no transient states populated (excitation beam off), we are able to measure the *transient* amount of total transmission. The recorded value ΔX is given by

$$\Delta X = \frac{X_1 - X}{X}$$

Increased transmission of the excited sample compared to the sample with just a ground-state population is indicated by positive values of ΔX.

The wavelength of the probe beam is adjusted in the main absorption band of the dye. Therefore the ground-state depletion causes an increase in the transmitted beam intensity. With respect to the high extinction coefficient of the ground state, the absorption of the excited singlet and the triplet state at this wavelength can usually be neglected. Thus information about the total yield of all populated transient states can be obtained.[10]

In Ref. 9 the sample is moved by using the jet technique. This technique restricts the amount of usable solvents to those showing a sufficient high viscosity. In order to be able to use solvents of any viscosity, the experimental setup was modified. Figure 2 shows the new experimental setup. Instead of the jet stream, the sample is located in a rotating dye cell, which is made of fused silica. Two dye solutions can be filled into two

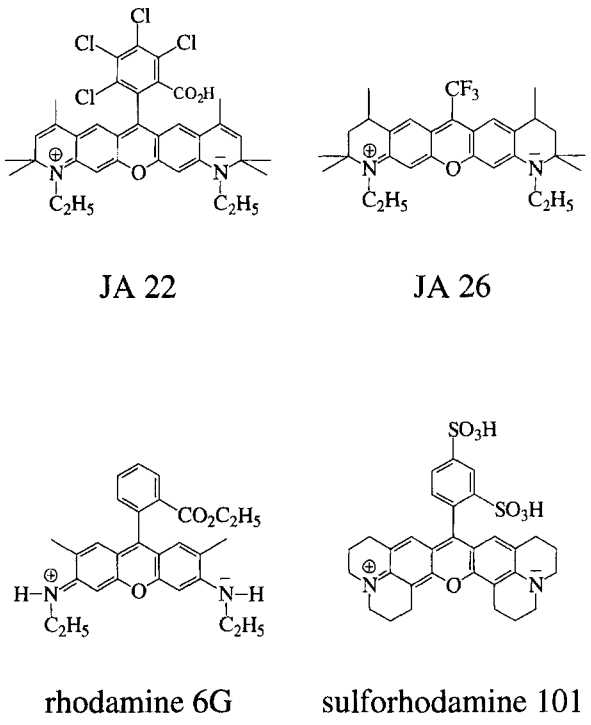

Fig. 3. Structures of the dyes used. Counterions: rhodamine 6G, chloride; JA 22 and 26, perchlorate.

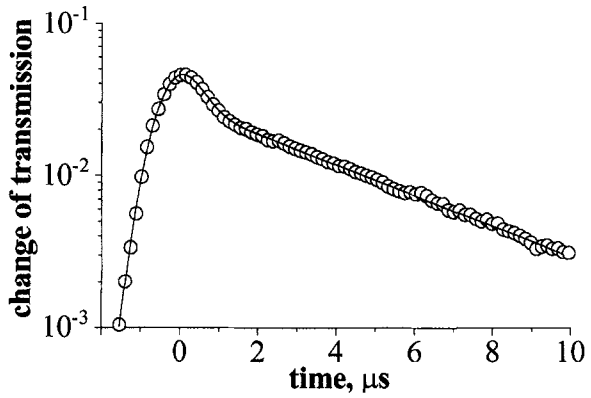

Fig. 4. Ground-state bleaching of rhodamine 6G in ethylene glycol. Excitation wavelength = 530.9 nm; probe wavelength and power = 514.5 nm and 3 mW; sample velocity = 14.3 m/s; absorbance at excitation wavelength = 1; absorbance at probe wavelength = 0.5; path length = 0.2 mm. (○) Measured signal; excitation power = 90 mW. (——) Theoretically fitted signal; τ_T = 4.4 μs; k_{ST} = 0.83 μs^{-1}; diameter of excitation and probe laser beam focus = 22 μm.

concentric tracks (200-μm thickness) residing in the cell. The system is driven by an electric motor (Type SA 2444 S BL2; Faulhaber), enabling it to rotate at a frequency up to 160 Hz, resulting in a maximum sample speed of 70 m/s.

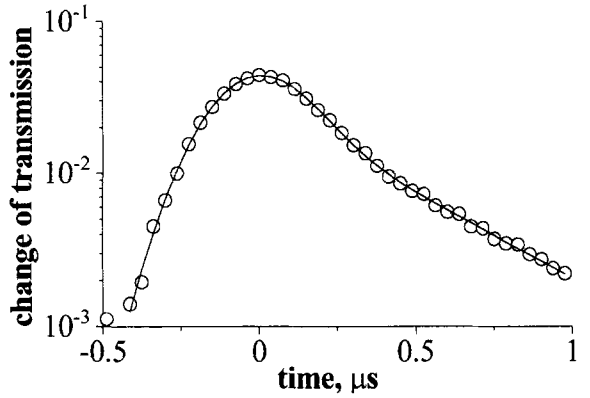

Fig. 5. Ground-state bleaching of rhodamine 6G in ethanol. Excitation wavelength = 530.9 nm; probe wavelength and power = 514.5 nm and 3 mW; sample velocity = 53.3 m/s; absorbance at excitation wavelength = 1.1; absorbance at probe wavelength = 0.6; path length = 0.2 mm. (○) Measured signal; excitation power = 80 mW. (——) Theoretically fitted signal; τ_T = 0.39 μs; k_{ST} = 2.3 μs^{-1}; diameter of excitation and probe laser beam focus = 23 μm.

We examined four dyes: rhodamine 6G (Radiant Dyes; laser grade), sulforhodamine 101 (ACROS; laser grade, free acid), JA 22, and JA 26 (synthesized and purified in the lab of Prof. Dr. Drexhage, University of Siegen). The structures are shown in Fig. 3. JA 22 and JA 26 were measured in ethylene glycol (Merck, p.a.) and ethanol (distilled). Experiments with rhodamine 6G and sulforhodamine 101 were also carried out in water (tridistilled). Trifluoracetic acid (Solvay) was added to solutions of JA 26 and JA 22 (2 ml/100 ml solution), whereas in the case of sulforhodamine 101, the addition was triethyl amine (Merck-Schuchardt; >99%; 2 ml/100 ml solution). All solvents used in the experiments were air-equilibrated at a temperature of 20°C. All dye solutions were filtered with polytetrafluorethylene membrane filters (Macherey & Nagel; pore diameter, 0.45 μm). The dye concentrations were adjusted in the range of 10^{-2} to 10^{-1} m*M*. Dependent on the investigated solution, the rotation frequency was between 30 and 130 Hz, giving sample velocities in the range of 12 to 55 m/s.

By varying the distance *D* between the excitation and the probe foci, the time evolution of the triplet population can be detected. The diameter of the excitation and detection laser beam (ca. 15 μm) and the speed of the sample restrict the time resolution to about 100 ns. For *D* = 0 (complete overlap between excitation and probe beam), the dye solution is bleached by population of the S_1 and T_1 state. Because of the short lifetime of the S_1 state (<10 ns), the ground-state depletion for *D* > 30 μm (no overlap between excitation and probe

Table I. Dynamic Data of the Triplet States

	Ethylene glycol			Ethanol			Water		
	k_{ST} μs^{-1}	τ_T (μs)	$k_{ST}\cdot\tau_T$	k_{ST} μs^{-1}	τ_T (μs)	$k_{ST}\cdot\tau_T$	k_{ST} μs^{-1}	τ_T (μs)	$k_{ST}\cdot\tau_T$
JA 22	1.7	5.8	9.9	1.6	1.1	1.8	—	—	—
JA 26	0.32	4.4	1.4	0.71	0.55	0.4	—	—	—
Srh 101[a]	0.37	9.0	3.3	2.4	0.63	1.5	0.32	4.8	1.5
Rh 6G[a]	0.83	4.6	3.8	2.3	0.39	0.9	0.88	1.9	1.7

[a]Srh 101, sulforhodamine 101; Rh 6G, rhodamine 6G.

beam) is determined exclusively by the population of the T_1 states. By comparison of the signals at $D = 0$ and $D > 30$ μm, the intersystem crossing rate k_{ST} can be calculated. With our experimental setup it is possible to determine triplet quantum yields down to 10 ppm. A detailed description of the data analysis is given in Ref. 10.

RESULTS AND CONCLUSION

As mentioned above, the product of the rate k_{ST} of intersystem crossing and the time decay τ_T of the triplet state can be of great importance for the fluorescence intensity. Hence it was the aim of our measurements to determine these parameters for different dyes in various solvents. This can be done by evaluating the measured signals as described in detail elsewhere.[10] In Figs. 4 and 5 typical results are shown. It can be seen that the experimental data are in good agreement with the theory. The results of all measurements are depicted in Table I. This shows that the values of the product $k_{ST}\cdot\tau_T$ can be quite small compared to unity, as in the case of JA 26 in ethanol, as well as significantly greater than 1, e.g., for JA 22 in ethylene glycol. Thereby JA 22 is the dye which shows the most explicit solvent dependence. The connection of the determined values of $k_{ST}\cdot\tau_T$ to the abscissa of the theoretically curve calculated by Eq. (5) is shown in Fig. 1. As shown in Fig. 1 even for frequently used dyes the triplet-state population may significantly reduce the fluorescence intensity.

The structure of the molecules as well as the solvent clearly affects the extent of the fluorescence reduction by the population of the triplet state. The solvent influence on the values of k_{ST} and τ_T can be qualitatively explained by the solubility and diffusion of oxygen in the solvents used.[10] So far the correlation between the chemical structure and the triplet-state parameters is unknown.

Considering our results the quantum yield of fluorescence cannot be the exclusive criterion for choosing a suitable dye, because the triplet-state properties can strongly decrease the obtainable fluorescence.

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