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Self-quenching of uranin: Instrument response function for color sensitive photo-detectors

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Abstract

Concentration is a key determining factor in the fluorescence properties of organic fluorophores. We studied self-quenching of disodium fluorescein (uranin) fluorescence in polyvinyl alcohol (PVA) thin films. The concentration dependent changes in brightness and anisotropy were followed by a lifetime decrease. We found that at a concentration of 0.54 M, the lifetime decreases to 7 ps. At a concentration of 0.18 M the lifetime was 10 ps with the relatively high quantum yield of 0.002. In these conditions the fluorescence intensity decay was homogeneous (well approximated by a single lifetime). We realized that such a sample was an ideal fluorescence lifetime standard for spectroscopy and microscopy, and therefore characterized instrument response functions for a time-domain technique. We show that self-quenched uranin enables measurements free of the color effect, making it a superior choice for a lifetime reference over scattered light.

Keywords

Self-quenching; Homotransfer; Homo-FRET; Instrument response function; Lifetime standard; Single photon avalanche diode

1. Introduction

Since the establishment of fluorescence studies as an independent area of spectroscopy, fluorescein has been among the most thoroughly studied organic dyes, chiefly because of its exceptional photophysical properties: high extinction coefficient and quantum yield as well as green-yellow spectral region which enabled sensitive visual detection. Fluorescein also displays a very high solubility in water, as well as pH sensitivity. The absorption and fluorescence spectra of fluorescein strongly overlap, which makes this compound an ideal model for an energy migration (homotransfer) study. Recently, fluorescence anisotropy

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We dedicate this paper to Professor Afons Kowski on the occasion of his 85th birthday.

changes that accompany the energy migration process have been studied experimentally and modeled with the Monte Carlo method [1].

The energy migration process often results in self-quenching. In the case of fluorescein and its derivatives the concentration dependent quenching is very strong [2,3]. However, most studies were done on a steady-state fluorescence intensity and anisotropy. The purpose of this study was to measure the lifetime changes of fluorescein emission during the self-quenching process, the goal being to see if quenched samples can be used as lifetime standards.

The time-resolved fluorescence technique is used in biology, chemistry and physics to study the fluorescence decays of electronically excited molecules. However, due to the excitation pulse duration and time resolution of the measurement system, the instrument response function (IRF) must be taken into account. In practice, this is accomplished by replacing the fluorescence sample with a scattering medium and recording its Rayleigh component. Unfortunately, this simple procedure is not applicable for all the spectroscopic/microscopic systems and their detection parts. Presently, the obstacle is the laser blocking dichroic beam splitters, filters and monochromators which have to be readjusted to measure $\lambda_{\text{ex}} = \lambda_{\text{obs}}$. Another issue arises if one uses photomultiplier tubes (PMT) and/or avalanche photodiodes (APD) for signal detection. Their timing characteristics (transit, rise time and transit time spread) are very sensitive to the wavelength of light incident on the photosensitive area (energy of detected photon). This property (also-called the color effect) is characterized by differences in shape and duration of IRFs. The response (for PMT and APD) recorded in the $\lambda_{\text{ex}} \neq \lambda_{\text{obs}}$ is not identical with the response at the normal detection wavelength [4,5]. For high speed detectors like microchannel plate photomultipliers (MCP PMT) or streak cameras, the variation is apparently negligible [6]. However, these detectors are less popular because of lower detection sensitivity and damage threshold as well as high cost. For these reasons cheaper PMTs and APDs are the most widely used detectors in the field of spectroscopy/microscopy. There is a strong demand for good lifetime standards capable of determining the IRFs in the required spectral region. The idea of using fluorescence standards for IRF is not new. Over the last several decades, many different IRFs were proposed and widely used in time-resolved frequency and time-domain techniques. Two groups originally proposed using a fluorophore for a correct instrument profile: Shabo's [7] and De Schryver's [8]. The other proposed methods are based mainly on dynamic quenching of the fluorophore's lifetime with an exterior quencher [9–12]. Also, the natural properties of the dyes with characteristically short lifetime in proper environments have been very often exploited. Erythrosine B in water served as IRF (with lifetime about 80 ps) to extract a proper fit procedure to the intensity decays of flavins [13], LDS798 in water (lifetime of 24 ps) as IRF in NIR spectroscopy [14]. In the ultraviolet spectral region, short lifetime high quantum yield fluorophores such as oxazoles were characterized as lifetime standards [15]. Styrenes and stilbenes were also proposed as picoseconds standards [16–18]. The summarizing overview of the methods used for correct evaluation of intensity decays in time-resolved spectroscopy was done recently by Boens et al [19].

Here we propose a new procedure to correct the IRF artifact resulting from the color effect. The procedure is based on fluorescence concentration quenching of disodium fluorescein (uranin) that demonstrates extremely short lifetime in picosecond range. The self-quenched samples are portable and easy to handle, have relatively high brightness, high time stability and can be used effectively in spectroscopy and microscopy in the most valuable visible region (500–550 nm).

2. Materials and methods

2.1. Chemicals

Poly(vinyl) alcohol (PVA) 99+% ($M_w = 146000\text{--}186000$), erythrosine B (ErB) and spectral grade ethanol were purchased from Sigma-Aldrich, St. Louis, MO. The uranin (disodium fluorescein) and rhodamine B (RhB) were obtained from Lambda-Physics, Ft. Lauderdale, FL. All compounds were used without further purification. Water used for films preparation was deionized using the Milli-Q Synthesis A10 system produced by Millipore.

2.2. Sample preparation

PVA (16 mg) was dissolved in 184 ml of water and boiled for 15 min. After cooling at room temperature, the PVA solution was divided among equal fractions and a relevant amount of fluorescein was added to them. The mixture was stirred at room temperature for 10 min. The solution was spin-coated using Special Coating Systems, Inc. Model P6700 series, at 3000 rpm for 100 s onto laboratory cover slips and left for 24 h to remove the solvent. A sub-micron thickness of the films ($0.6\text{ }\mu\text{m}$) was too small for direct measurements; therefore, we measured absorptions of the samples. Next, we estimated the thickness of the spin-coated samples (and uranin concentrations) by comparison of optical densities of the sample with the reference film of known thickness and known uranin concentration.

The reference sample was prepared from a known concentration of uranin in PVA solution. We dried the reference sample in a Petri dish for 72 h. Then, uranin doped PVA film had been detached from the dish. We cut out a regular (rectangular) middle part of a film, measured its volume and weight, first separately, and next together with the weight of remaining parts of the films. Ratio-metric calculations of these values let us specify the concentration of the uranin dye in PVA after drying. From the absorption measurements, using the Lambert–Beer law, extinction coefficients for uranin in PVA were calculated and their average value was estimated to be about $55,000\text{ cm}^{-1}\text{ M}^{-1}$. This value was used for concentrations calculations of a set of spin-coated samples for the self-quenching study.

The films were stored at room temperature to be used for further measurements.

2.3. Steady state measurements

Absorption spectra were carried out with a Cary 50 Bio (Varian) in the range of 400–600 nm. Fluorescence spectra excited at $\lambda_{\text{ex}} = 470\text{ nm}$ were monitored with a Cary Eclipse spectrophotometer (Varian) in the range 480–650 nm in the front-face configuration, using a makeshift attachment.

2.4. Time-resolved fluorescence (lifetime) measurements

Fluorescence decays were measured with time-correlated single-photon-counting equipment (FluoroTime 200, Picoquant GmbH, Berlin, Germany) provided with monochromator, polarizers and a PMT MCP detector (Hamamatsu). Excitation was achieved with a pulsed 470 nm laser diode (40 MHz). The IRF was recorded with Ludox showing full-width half-maximum (FWHM) around 80 ps. This equipment with iterative deconvolution of the fluorescence intensity decays provided reliable and exceptional resolution in sub-nanosecond range. The samples were placed and measured under identical conditions (front face configuration) as in the steady state experiments. The time-resolved spectra were reconstructed from the decay curves using a sum of exponentials:

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i) \quad (1)$$

where α_i and τ_i are pre-exponential factors and fluorescence lifetimes, respectively. The FluoFit software package (version 4.4) was used to describe the data.

2.5. Time-resolved fluorescence (microscopy) measurements

Time resolved fluorescence microscopy measurements were performed on a MicroTime 200 (Picoquant Inc. GmbH, Berlin, Germany). The measuring setup previously described [20] is summarized here briefly: the laser beam was coupled into a microscope objective with a high numerical aperture (1.2 water immersion, Olympus), which focused the beam onto the fluorescein doped PVA sample. The emerging fluorescence was collected by the same objective and separated from the excitation beam by the dichroic mirror (z638rpc, Chroma Technology Corp.). An additional long wavelength spectral filter for scattering suppression (HQ500lp, Semrock) was used. The fluorescence light was focused onto a confocal aperture, and detected by a single photon avalanche photodetector (SPAD) (SPCM-AQR-14, Perkin Elmer, Inc.). The intensity and lifetime images were achieved with a precise x-y piezo-scanner (model P-733.2CL, Physik Instrumente (PI) GmbH Co., Germany). The intensity decays were analyzed in terms of a multi-exponential model using SymPhoTime v. 5.0 software (PicoQuant, Inc. GmbH, Berlin). The amplitude average lifetime was calculated as

$$\bar{\tau} = \sum_i \alpha_i \tau_i \quad (2)$$

3. Results and discussion

3.1. Steady-state fluorescence measurements

Fig. 1 shows normalized absorption and fluorescence spectra of uranin doped PVA film. A quite large spectral overlap (shadowed area) strongly suggests the possibility of long range dipole-dipole coupling between molecules. The excitation energy localized on one molecule can be effectively transferred to another one in a Homo-FRET process [21–23]. Qualitative determination of the coupling strength is partially provided by overlap integral J according to

$$J(\lambda) = \frac{\int_0^\infty F_D(\lambda) \epsilon_A(\lambda) \lambda^4 d\lambda}{\int_0^\infty F_D(\lambda) d\lambda} \quad (3)$$

where F_D is the fluorescence emission spectrum of the donor and ϵ_A is the molar extinction coefficient of the acceptor at the same wavelength (λ). On the basis of the overlap integral, the Förster distance (R_0) of a donor-acceptor pair can be calculated (Eq. 3) equal to the donor-acceptor separation at which FRET is 50% efficient:

$$R_0 = 8.79 \times 10^3 [Q_D \kappa^2 n^{-4} J(\lambda)]^{1/6} \quad (4)$$

where κ^2 is the averaged orientation factor, Q_D is the donor quantum yield (in our case 0.95), n is the index of refraction of PVA ($n = 1.5$). It is reasonable to assume the static relative dipole orientations of the donor and acceptor fluorophores in a disordered rigid PVA matrix, thus making $\kappa^2 = 0.467$ [20,24,25]. Determined value of R_0 was 45.2 Å, which is very close to the value estimated earlier [1].

Table 1 at the top of Fig. 2 shows the fluorescence intensity measurements of uranin-doped PVA samples spin-coated on microscope cover slips. For lower dye concentrations the

accuracy of the measurements dropped down due to a high error margin in the estimated absorptions; moreover, for concentrations higher than 1 mM, the dependence can be accurately recovered. Fluorescence anisotropies (Fig. 2, bottom; Table 1) are more precisely determined because the measurements are ratiometric and do not require measurements of individual absorptions. The fluorescence *anisotropies* drop substantially at 10^{-3} – 10^{-2} M concentration of uranin. Essentially, it is an effect of excitation energy homotransfer to molecule oriented and fluorescing in the other direction relative to absorbing one. At higher concentrations (above 135 mM) one can observe a repolarization effect. In our experiment we noticed that anisotropy from the lowest value (~ 0.007) increases up to (~ 0.11). The explanation of fluorescence repolarisation can be easily given in the case of monomers and non-fluorescent dimers. Initially, with the increase in the dye concentration, the concentration ratio of monomers to dimers is high, allowing effective energy homotransfer between monomers with almost no trapping by dimers. Thus, at sufficiently high dye concentration the average distance between monomers becomes short and the formation of dimers becomes effective. As each dimer is formed out of two monomers, the monomer to dimer concentration ratio decreases. In the language of energy transfer and trapping processes it means that at these concentrations energy homotransfer becomes weaker and is replaced by stronger trapping by dimers. Therefore, at high concentrations the rest of luminescing molecules consists mostly of initially excited monomers emitting polarized light.

3.2. Time-resolved measurements

3.2.1. Lifetimes of self-quenched uranin—We measured the lifetimes of all the samples used in steady-state study. Selected fluorescence intensity decays are presented in Fig. 3. The measured fluorescence intensity decays were analyzed with a multi-exponential model. The results of this analysis are presented in Table 2. Fig. 4 shows the plotted amplitude of averaged lifetimes as a function of uranin concentration. At extreme high and low concentrations, the decays are homogeneous and well approximated by a single lifetime. However, at intermediate concentrations the decays are heterogeneous and two or three lifetimes are needed to fit the data. There is no description of a homogeneity/heterogeneity measure in the literature. To measure heterogeneity, we propose to use the ratio of the quality of a single exponential fit to the best multi-exponential approximation:

$$\chi_r^2(1\text{ exp})/\chi_r^2(m\text{ exp}) \quad (5)$$

If this ratio is close to unity, the decay is homogeneous, higher numbers indicate higher degree of heterogeneity. Interestingly, the heterogeneity of our samples reaches a maximum at a uranin concentration of about 7 mM (Fig. 5, Table 2).

Next, we plotted a Stern–Volmer like dependence (Fig. 6) using amplitude averaged lifetimes [26]. This dependence suggests: (1) a dynamic character of the quenching and (2) highly sub-linear character of the plot indicates a complex quenching process. It is difficult to determine the mechanism of uranin self-quenching because such sub-linear dependence could be the result of many occurring processes like a simultaneous static quenching [27] or a bimolecular process of dimerization. In this manuscript, we focus on the utilization of ultra-short lifetimes rather than on the self-quenching mechanism.

3.2.2. Evaluation of IRF based on self-quenched uranin—We wondered if a highly self-quenched uranin sample can serve as an IRF at the emission wavelength. Using a MCPMT detector, free of color effect, we measured responses of scattered light at a 470 nm excitation wavelength (Fig. 7, top) and uranin fluorescence at 525 nm (Fig. 7, bottom).

As a reference for scattering we used a non-fluorescent microscope cover slip in order to match the experimental geometry (similar cover slips were used for the samples preparation). For the readers' convenience, we present in the right panels a linear version of IRFs. Both IRFs are almost identical with FWHM of about 104 ps. In the case of MCPMT detectors they can be used alternatively. However, in the case of color sensitive detectors the condition may be different.

3.2.3. Lifetime measurements with color sensitive detectors—We evaluated the IRFs described above with a MT200 time-resolved microscope equipped with an APD detector (Fig. 8).

Both response functions (for scattered light and fluorescence of self-quenched uranin) were recorded using a 470 nm excitation wavelength and the same detector. The only difference is in the observation: 470 nm for scattered light and above 500 nm for the self-quenched uranin. The difference in wavelength observation strongly impact the FWHMs of the response pulses (568 ps for $\lambda_{\text{ex}} = \lambda_{\text{obs}}$ and 420 for $\lambda_{\text{ex}} \neq \lambda_{\text{obs}}$). The broadening of the IRF at shorter wavelengths can be explained by the secondary photoelectron processes. This is unavoidable in the case of APD and PMT detectors. Of course, the problem will be resolved if both, IRF and studied fluorescence are detected exactly at the same wavelength.

Wondering how strongly the unmatched IRF will affect the measured fluorescence decays, we selected fluorophores with known homogeneous decays, Erythrosine B in methanol and Rhodamine B in water [19,28]. The lifetime measurements and analyses are presented in Figs. 9 and 10. In both cases the fits with using IRF based on scattered light were poor and suggest another artificial component (Table 3). On the other hand, the measurements and analyses with proposed self-quenched uranin as a reference revealed accurate expected lifetimes.

4. Conclusions

We have demonstrated how to manufacture and use samples (dye-doped PVA films) for IRFs, based on Homo-FRET (self-quenching) effect. A high efficiency of uranin self-quenching results in extremely short fluorescence lifetimes, shorter than 10 ps. Although the lifetime of quenched uranin is very short, the brightness is relatively high, and the fluorescence intensity decay is homogeneous, well approximated with one lifetime. We recommend this ultra-short reference standard in a green spectral region for a time-resolved fluorescence spectroscopy and microscopy equipped with avalanche photodiode detectors or photomultipliers. The use of self-quenched uranin-doped PVA samples is very convenient and avoids potential errors in decay analysis.

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Fig. 1.

Absorption and fluorescence spectra of uranin doped PVA film. Fluorescence spectrum was recorded upon excitation at 470 nm. The shaded area indicates the spectral overlap between the emission and the absorption spectra. The strong spectral overlap results in high Förster distance R_0 . Top panel shows a photograph of one of the sample used, 0.18 M of uranin doped PVA, spin-coated on a 25 mm $\times$ 25 mm microscope cover slip.

Fig. 2.

Top: steady state emission intensities of uranin doped PVA films. The measurements were done in front-face geometry and the intensities were corrected for samples optical densities. Bottom: concentration dependent emission anisotropy of uranin. The anisotropy decrease is followed by a repolarization at highest uranin concentrations.

Fig. 3. Time-domain responses of progressively self-quenched emission of uranin-doped PVA, spin-coated on microscope cover slips.

Fig. 4. Amplitude averaged lifetimes of uranine doped PVA in function of the concentration of the dye.

Fig. 5.

Homogeneity parameters ($\chi_R^2(1 \text{ exp.})/\chi_R^2(m \text{ exp.})$, see text) of fluorescence intensity decays in function of the uranin concentration. At low and high concentrations the decays are homogeneous.

Fig. 6. Self-quenching of uranin in PVA films. The lifetime changes suggest a dynamic process. A sub-linear character of the quenching indicates a complex mechanism.

Fig. 7.

Top: IRF obtained with a scattered light, detected at 470 nm. Bottom: the fluorescence intensity decay of the sample containing 0.18 M uranin. The observation was at 525 nm. A 470 nm excitation laser and MCP PMT detector were used in both experiments. The right panels present IRFs in linear scale of intensities to visualize details. The FWHMs of both pulses are approximately 104 ps.

Fig. 8.

IRFs obtained with (a) scattering medium, detected at 470 nm, (b) fluorescence of the self-quenched sample of uranin (0.18 M) measured at >500 nm. The same 470 nm pulsed laser was used for the excitation of both samples, and the same Perkin Elmer detector was used for the observation. There is a significant difference in FWHMs pulses.

Fig. 9.

The fluorescence intensity decays of erythrosine B (ErB) in ethanol analyzed by using the instrumental response function obtained for (a) Rayleigh scattering and (b) fluorescence of self-quenched uranin reference. Both functions were recorded for 470 nm pulsed laser excitation and detected using Perkin-Elmer SPCM-AQR-14 detector. The bottom panels of the intensity decays display the residuals. A poor fit to the data ($\chi^2 = 19.1$) was achieved with the scattered light while satisfactory approximation was obtained with the reference lifetime standard.

Fig. 10.

Time-resolved fluorescence of rhodamine B (RhB) in water analyzed by using the IRFs: (a) obtained at 470 nm using scattered light and (b) as a fluorescence of the self-quenched uranin reference. The samples were excited by 470 nm pulses at 40 MHz repetition rate. Both IRF functions were recorded by Perkin-Elmer SPCM-AQR-14 detector. The bottom panels of the intensity decays display the residuals. The fit with using the scattered excitation light (top) failed to satisfactorily resolve the decay.

Table 1

Emission intensity and anisotropy analysis of different concentrations of uranin doped PVA films.

Concentration [mM]	Emission intensity	Anisotropy
540	0.060	0.108
180	0.189	0.08
135	0.351	0.074
90	2.944	0.07
54	2.801	0.068
18	2.124	0.067
10.8	7.057	0.073
8.1	9.139	0.09
7.2	10.676	0.101
3.6	37.833	0.207
1.8	90.333	0.346
0.18	114.162	0.383
0.09	130.239	0.385
0.072	90.538	0.385

Table 2

Multi-exponential analysis of fluorescence intensity decays of uranin doped PVA films.

Concentration [mM]	a_1	τ_1 (ns)	a_2	τ_2 (ns)	a_3	τ_3 (ns)	a_4	τ_4 (ns)	$\bar{\tau}^a$ (ns)	$\langle \tau \rangle^b$ (ns)	$\chi^2_{\text{R}}(1\text{exp.})/\chi^2_{\text{R}}(\text{m.exp.})$
540	1	0.007							0.007	0.007	1.75/1.75
180	1	0.010							0.010	0.01	1.26/1.26
135	0.002	0.202	0.998	0.011					0.018	0.011	2.1/1.13
90	0.001	0.623	0.995	0.011	0.004	0.169			0.030	0.012	3.64/0.97
54	0.001	0.632	0.014	0.158	0.985	0.014			0.058	0.017	4.84/0.99
18	0.004	0.767	0.101	0.154	0.895	0.046			0.115	0.06	7.72/0.87
10.8	0.002	1.622	0.045	0.475	0.600	0.178	0.353	0.046	0.236	0.147	10.42/0.86
8.1	0.014	1.032	0.469	0.355	0.517	0.149			0.332	0.258	14.57/0.89
7.2	0.056	1.251	0.435	0.533	0.509	0.202			0.573	0.405	27.15/0.91
3.6	0.202	1.942	0.567	1.171	0.231	0.321			1.383	1.129	7.42/0.96
1.8	0.681	2.908	0.319	1.301					2.629	2.394	3.5/1
0.18	0.77	3.945	0.23	3.361					3.829	3.813	1.09/0.92
0.09	1	3.75							3.89	3.89	1.04/1.04
0.072	1	3.91							3.91	3.91	0.99/0.99

The excitation at 470 and the observation at 525 nm.

$$\bar{\tau}^a = \frac{\sum_i f_i \tau_i}{\sum_i f_i}, f_i = \frac{\alpha_i \tau_i}{\sum_i \alpha_i \tau_i}$$
$$b\langle \tau \rangle = \sum_i \alpha_i \tau_i$$

Table 3
Time resolved fluorescence data analysis of ErB and RhB with different IRFs.

Compound	IRF	a_1 (ns)	τ_1 (ns)	a_2 (ns)	τ_2 (ns)	$\bar{\tau}$ (ns)	$\langle \tau \rangle$ (ns)	χ^2_R
ErB in MeOH	Scatterer	1	0.43			0.43	0.43	19.1
	Scatterer	0.9	0.03	0.1	0.49	0.34	0.07	1.4
	Quenched uranin	1	0.48			0.48	0.48	1.1
RhB in water	Scatterer	1	1.53			1.53	1.53	9.2
	Scatterer	0.86	0.04	0.16	1.59	1.41	0.25	1.1
	Quenched uranin	1	1.58			1.58	1.58	1.3