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The photophysics of Lissamine rhodamine-B sulphonyl chloride in aqueous solution: implications for fluorescent protein–dye conjugates

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Abstract

The absorption, emission and fluorescence excitation spectra, fluorescence quantum yields and fluorescence lifetimes of Lissamine rhodamine-B sulphonyl chloride (LRSC) dye have been measured as a function of pH and concentration in air-saturated and degassed aqueous solutions and in acetone, and the spectra measured at both 295 and 80 K. A single chemical species, the zwitterion, is responsible for both absorption and emission at all pH's examined. With excitation in the dye's strong S_1–S_0 absorption system in the visible, the quantum yield of emission in aqueous media is 0.33, independent of excitation wavelength, and the excited state decays monoexponentially with a lifetime of 1.63 ns. Oxygen in air-saturated solutions, and dye concentration up to 0.1 mM have no effect on the photophysics, and no phosphorescence from degassed frozen solutions is observed. These results and literature data are used to determine the stoichiometry of immunoglobulin–LRSC dye conjugation using capillary electrophoresis and a fibre optic-based fluorescence detector.

Keywords: Photophysics; Lissamine rhodamine-B sulphonyl chloride; Fluorescent protein–dye conjugates

1. Introduction

Fluorescent dyes are widely employed in imaging applications and as molecular probes in biological and biomedical science. Frequently, it is not necessary to know the details of the photophysics and spectroscopy of the dyes employed for these purposes; the absorption and emission spectra and a measure of relative emission intensity are the only data needed [1]. Increasingly, however, it has become necessary to have more quantitative information about the behaviour of the fluorophore in bound and unbound chemical forms, for example, when quantitative correlation methods are used in fluorescence microscopy or when fluorescent probes are used for single molecule detection [2–4].

Rhodamine dyes, in various chemical and physical formulations, are often used for these purposes [5]. Rhodamine's sulphonyl halide derivatives are water soluble and react with proteins by acylation of free amino groups at their N-termini or in amino acid residues such as lysine. Reactions with serine residues at the active sites of some enzymes are also well documented. The sulphonamide products of these sulphonyl halide–amine reactions are highly fluorescent and stable under the hydrolysis conditions used for amino acid analysis. The rhodamine sulphonyl halides can also be used as polar fluorophores for labelling phospholipids which have an amine terminus in their head group, whereas rhodamine's amine and hydrazine derivatives can be used to label proteins at accessible aldehyde and ketone moieties [3].

One of the commonly used water soluble forms of rhodamine dye is the ethanaminium diethyl amino sulphonyl chloride derivative, which is sold under the trademarked name Lissamine rhodamine-B sulphonyl chloride (LRSC, structure in Scheme 1). Together with its tetramethyl and Texas Red® analogues, this dye has been subjected to routine spectroscopic and brief photophysical investigations [6–9], but has not been examined systematically to determine its properties under the range of conditions which might be experienced in common use. Nominally, this fluorophore is sold as the 9-[4-chlorosulphonyl-2-sulphophenyl]-derivative, but this major component can be contaminated by other isomers. Differences in the spectroscopic and photophysical behaviour of LRSCs fluorescent sulphonamide derivatives have been attributed to the contaminating 9-[2-chlorosulphonyl-4-sulphophenyl]-isomer [8].

The spectroscopic and photophysical properties of the major isomer of LRSC are reported in this paper and the implications of these measurements on the use of LRSC as a fluorescent label for proteins and phospholipids are discussed.

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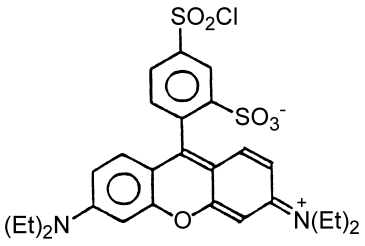

Scheme 1. Structure of LRSC; 9-[4-chlorosulphonyl)-2-sulphophenyl]-3,6-bis(diethylamino)-xanthylium hydroxide inner salt.

2. Experimental

Absorption spectra were taken on Cary 1E and Varian-Cary 100 spectrophotometers. Emission and emission excitation spectra were recorded using a Spex Fluorolog 212 spectrofluorometer, and were corrected for variations in the sensitivity of the instrument's detection and excitation systems with wavelength. Spectra taken at liquid nitrogen temperature were obtained using an Oxford instruments cryostat.

Fluorescence lifetimes were measured by time correlated single photon counting using a synchronously pumped, mode-locked argon ion-dye laser system which was cavity dumped at 4 MHz. Typically this system provides excitation pulses <10 ps in width spaced 250 ns apart, and exhibits excellent rejection of pre- and post-mode-locked pulses at 12 ns intervals. Excitation at 580 nm was provided by the fundamental of rhodamine 6G dye, whereas excitation at 345 nm was provided by frequency-doubling the output of DCM dye. To avoid rotational depolarization artefacts, fluorescence was observed through a polarizer set at the magic angle (54.7°) relative to the plane of polarization of the excitation light. It was placed in front of a Zeiss M4 quartz prism monochromator used to select the emission wavelength and an RG 630 cut-off filter used to reject scattered exciting light.

The detector was a Hamamatsu R2809U-07 microchannel plate photomultiplier tube cooled to −30°C and operated at 3100 V. Signals from the MCP-PMT were fed to a photon-counting system of standard design operating in the reverse start–stop mode. Count rates were kept below 1 kHz to avoid pulse pile-up artefacts. The details have been given elsewhere [10,11].

Instrument response functions were recorded by observing scattered excitation light. Fluorescence decay constants were obtained by iteratively convoluting trial decay functions with the measured instrument response function and comparing the result with the measured temporal fluorescence decay profile. In all cases in which fluorescence was excited directly in the S_1–S_0 absorption system, the decays were well-represented by single exponential functions, as judged by the reduced chi-squared value (χ^2), visual inspection of the distribution of weighted residuals and other statistical measures of the "goodness-of-fit".

Fluorescence quantum yields were measured by a relative method using rhodamine 101 which has a fluorescence quantum yield of 1.00 in ethanol at room temperature [12] as the standard. Care was taken to ensure that absorbances of the sample and standard were closely matched and kept below 0.1 to avoid geometric and inner filter artefacts, and "n^2" corrections were applied to correct for differences in the refractive indices of the sample and the standard [13]. Fluorescence intensities were obtained by integrating the corrected emission spectra over their full spectral range. Measurements were repeated up to 10 times to ensure good statistical significance.

Separation of LRSC from its protein conjugates was achieved by capillary electrophoresis (CE), and fluorescence detection and quantitation was achieved with a fibre optic system based on a design of Bruno et al. [14]. A 363 μm o.d., 75 μm i.d. fused-silica capillary (Polymicro Technologies) of 60 cm length was suspended between running aqueous buffer solutions consisting of 10 mM borate ($Na_2B_4O_7{\cdot}10H_2O$) and 0.5 mM sodium dodecyl sulphate. The separation voltage was typically 20 kV and injection was provided by a standard gravitational technique. Two 100 μm optical fibres, one for laser excitation and a second to gather fluorescence, were attached by refractive index-matching epoxy resin to the capillary at a point 45 cm from the injection end. Excitation was provided by coupling the output of either a HeNe laser at 543.5 nm (through an interference filter) or, for higher powers, an argon ion laser at 514.5 nm through a fibre optic coupler (Spindler and Hoyer) containing a graded refractive index lens. Emission was collected at right angles to excitation and was detected by feeding the optical fibre into the Spex fluorometer and using the instrument in its standard photon counting mode. Cut-off filters were used to reject scattered light. With this system, the limit of detection was <1 fmol and the response (fluorescence peak area) was linear over the entire range of LRSC concentrations $< 1 \times 10^{-3}$ M and argon ion laser excitation powers <800 mW.

LRSC was obtained from Molecular Probes and was purified by recrystallization and column chromatography. Water was purified by distillation and passage through a Millipore filtration system and exhibited almost no residual fluorescence at any excitation wavelength in the UV–visible range. Organic solvents and buffer components were of highest available purity and were used as received. Unlabelled human immunoglobulin (IgG_1) was obtained from Chemicon International and was used as received. LRSC–IgG conjugates were prepared as described by the supplier of LRSC, Molecular Probes.

3. Results and discussion

The absorption and fluorescence emission spectra of LRSC in aqueous solution at room temperature are shown in Fig. 1, and the absorption and normalized, corrected fluorescence excitation spectra are compared in Fig. 2. These spectra are all independent of the concentration of the dye up to

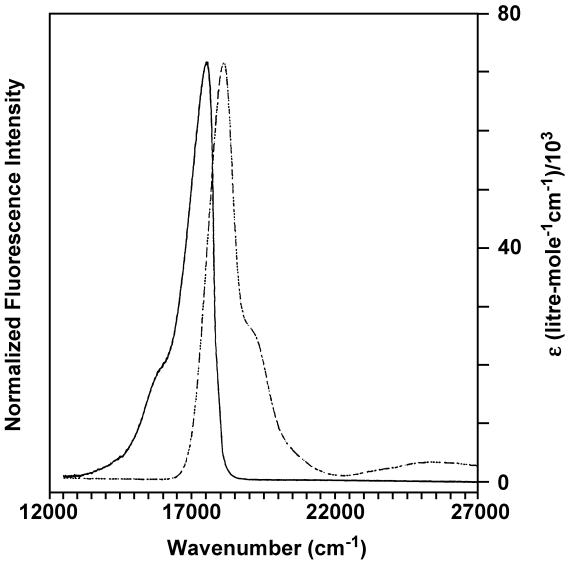

Fig. 1. Absorption (dashed) and normalized emission (solid) spectra of LRSC in air-saturated aqueous solution at pH 5.37.

0.1 mM and acidity in the range $1 < \text{pH} < 12$. The maxima of the absorption and emission spectra are located at 564 and 583 nm, respectively, $\varepsilon_{max} = 7.15 \times 10^4\,M^{-1}\,cm^{-1}$, the absorption and corrected excitation spectra are identical between 450 and 700 nm, and the absorption and emission spectra, which are near perfect mirror images, exhibit a small Stokes shift of 580 cm^{-1}. The fluorescence spectrum is very clean at $\lambda < 550$ nm, unlike a previously published spectrum [6] which exhibits significant emission to the blue of the onset shown in Fig. 1. In deoxygenated ice at ca. 80 K, the emission spectrum (not shown) shifts 4.5 nm to the blue, sharpens somewhat and reveals more vibrational structure, but shows no evidence of a longer wavelength emission which might be attributed to a phosphorescent triplet. In this low temperature fluorescence spectrum, spacings of 1345 and 2940 cm^{-1} are found between the emission maximum at 578.5 nm and weaker vibrational features at 627.3 and 697 nm. These wavenumbers correspond well with intense features in the IR spectrum of a solid sample of LRSC at 1339 and 2945 cm^{-1}.

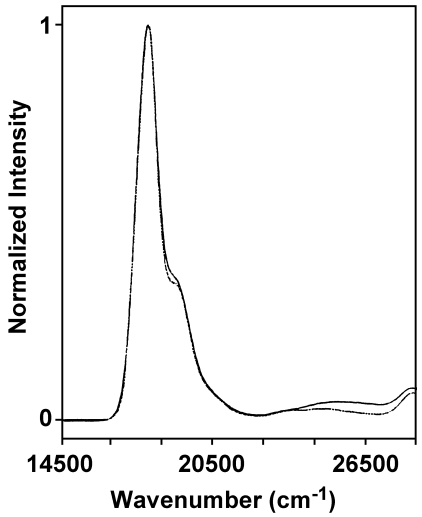

Fig. 2. Absorption and normalized fluorescence excitation spectra of 1.0×10^{-5} M aqueous LRSC at pH 5.37. The slight difference between the two spectra near 19 000 cm^{-1} can be attributed to the use of a larger spectral bandwidth in the excitation spectrum.

These spectroscopic data suggest that the ground, S_0, and fluorescent excited, S_1, states of LRSC are similar in structure, as expected given the highly conjugated nature of the chromophore, and that a single pH-insensitive chemical species is responsible for both absorption and emission. The coincidence of the absorption and excitation spectra in the 450–700 nm range suggests that there is no change in the quantum yield of emission for excitation over the entire breadth of the strong S_1–S_0 absorption system. However, at $\lambda_{ex} < 450$ nm the corrected, normalized excitation spectrum has an intensity (I_{ex}) which is lower than that of the absorption spectrum (I_{abs}), indicating that the quantum yield of fluorescence is smaller where excitation initially populates higher singlet states. This is indicative of the onset near 23 500 cm^{-1} of an upper state radiationless decay process which competes with internal conversion to S_1 and which has a quantum yield of $1-I_{ex}/I_{abs}$ (cf. Fig. 2). This observation is important in interpreting the photophysics of LRSC when it is excited at wavelengths in the UV (vide infra).

The excited state lifetimes of LRSC, τ, were determined in air-saturated and degassed pure water, in acidic and basic aqueous solutions in the range $0 < \text{pH} < 12$, in a 10 mM borate buffer at pH = 9.1, and in acetone. Most experiments were done at an excitation wavelength of 580 nm and emission was viewed at 630 nm through a quartz prism monochromator with a spectral bandwidth of 18 nm. All of the experiments employing S_1–S_0 excitation of aqueous and acetone solutions of LRSC yielded temporal fluorescence intensity profiles which could be fit using a single exponential decay function. A few experiments were done using an excitation wavelength of 345 nm, but these fluorescence decays were non-exponential. A typical log-linear plot of fluorescence intensity vs. time is shown in Fig. 3 for 7×10^{-7} M LRSC in degassed water at room temperature. The data are recorded in Table 1.

These data reveal a number of important features concerning the use of LRSC as a fluorescent probe. First, there is no evidence of any contamination by isomers of the dye that have photophysical or spectroscopic properties different from those of the nominal major component, the 9-[4-chlorosulphonyl-2-sulphophenyl]-derivative (Scheme 1). The potential contaminating isomers either have the same photophysical and spectral properties as the major component, or are present in quantities which are too small to detect by these methods. Second, there is no evidence of any effect of pH in the range $1 < \text{pH} < 12$ on the spectra or the excited state lifetime of the flurophore in aqueous media; a single chemical species is responsible

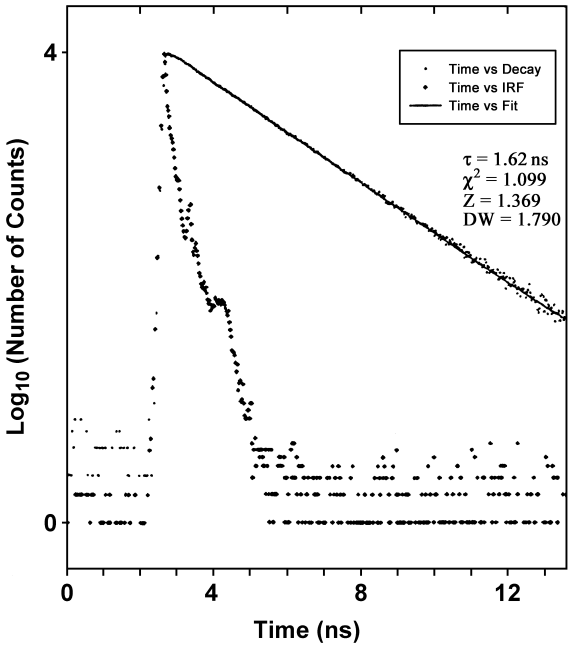

Fig. 3. $\log_{10}$(counts per channel) vs. time for the fluorescence of 7×10^{-7} M aqueous LRSC at pH 5.37. The measured fluorescence profile is given by the dotted line (···). The solid line gives the best fit from the iterative reconvolution of a single exponential decay function ($\tau = 1.62$ ns) with the measured instrument response function (+++).

for both absorption and emission. This is consistent with what is known about the aqueous acid–base chemistry of sulphonic acid derivatives of rhodamine dyes, and the prediction that LRSC will exist in one chemical form, that is shown in Scheme 1, over the entire $1 < \text{pH} < 12$ range. Third, oxygen has no effect on the spectra or the measured lifetimes, and the spectrum in degassed ice at liquid nitrogen temperature exhibits no additional emission. Although the quantum yield of non-radiative decay processes is ca. 0.67 (vide infra), either intersystem crossing to the triplet manifold is not important or any triplets that are formed do not exhibit significant emission. Fourth, the lifetimes and spectra of free LRSC are independent of ionic strength up to a modest concentration of 10 mM borate buffer (chosen because this is the medium for subsequent capillary electrophoresis of dye-conjugated protein). The fluorescence lifetimes are independent of the concentration of the dye at concentrations $<10^{-5}$ M, implying that both self-quenching and ground state aggregation are unimportant in aqueous media under these conditions. Finally, the lifetime of the S_1 state of 1×10^{-5} M LRSC in acetone is the same, within experimental error, as it is in a 1×10^{-5} M aqueous solution implying that the polarity and hydrogen-bonding capacity of such solvents do not have a measurable effect on the photophysics of the fluorophore.

Table 1
Fluorescence lifetimes of LRSC in several condensed media

Medium[a]	Number[b]	τ (ns)	χ^{2c}
H_2O, pH = 1	3	1.63 ± 0.01	1.17
H_2O, pH = 12	3	1.61 ± 0.01	1.14
H_2O, 10 mM borate	2	1.61 ± 0.01	0.99
H_2O, degassed	3	1.62 ± 0.01	1.12
H_2O, 1×10^{-5} M dye	3	1.67 ± 0.03	1.06
Global average, aqueous solution	14	1.63 ± 0.02	
Acetone, 1×10^{-5} M dye	3	1.68 ± 0.02	1.48

[a] [LRSC] = 7×10^{-7} M in room temperature air-saturated aqueous solution at pH = 5.37 unless otherwise noted.
[b] Number of determinations.
[c] Average for the listed number of determinations.

The quantum yields of S_1–S_0 fluorescence of dilute ($< 1 \times 10^{-6}$ M) solutions of LRSC were determined in air-saturated water at its natural pH of 5.37 and in acetone, both at room temperature. Repeated determinations of the quantum yields yielded values of $\phi_F = 0.33 \pm 0.01$ in water and $\phi_F = 0.35 \pm 0.05$ in acetone. The rate constants of radiative (k_r) and non-radiative ($\sum k_{nr}$) decay of the S_1 state of LRSC can be determined from these lifetime and quantum yield data using the standard relationships $k_r = \phi_F/\tau$ and $\sum k_{nr} = (1-\phi_F)/\tau$, where $\sum k_{nr}$ represents the sum of the rate constants of all parallel first order and pseudo-first order non-radiative processes by which S_1 is depopulated. The resulting values of these constants for aqueous solution are $k_r = 2.03 \times 10^8\ s^{-1}$ and $\sum k_{nr} = 4.10 \times 10^8\ s^{-1}$; the corresponding rate constants in acetone are not significantly different. The experimental value of k_r is in good agreement with a value of $k_r = 1.8 \times 10^8\ s^{-1}$, calculated from the oscillator strength of the S_1–S_0 transition using the procedure of Strickler and Berg [15]. Because neither oxygen nor self-quenching is observed, there is no contribution to $\sum k_{nr}$ from pseudo-first order kinetic processes. Thus $\sum k_{nr} = k_{ic} + k_{isc}$, the sum of the rate constants of S_1–S_0 internal conversion and S_1–T_1 intersystem crossing. In the absence of positive evidence of triplet formation, we assume $k_{ic} \gg k_{isc}$ and thus assign a value to $k_{ic} = 4.10 \times 10^8\ s^{-1}$.

A few excited state lifetime experiments were carried out using an excitation wavelength of 345 nm, but the resulting temporal fluorescence profiles could not be fit adequately using a single exponential decay function. These results are consistent with the observation that the quantum yield of fluorescence is lower when exciting in the S_n–S_0 ($n > 1$) absorption bands at $\lambda < 450$ nm than when exciting in the S_1–S_0 absorption system at $450 < \lambda < 600$ nm (cf. Fig. 2). The fact that complex decays are observed when upper excited singlets are initially populated suggests that a photoinitiated chemical reaction, rather than an unusual non-radiative photophysical decay path, is responsible. The most likely candidate is fast excited state photohydrolysis of the sulphonyl chloride group. A comparable photochemical reaction in protein–dye conjugates would be an unwanted complication in most fluorescence imaging or fluorescent probe applications. Thus it would seem prudent to excite only in the S_1–S_0 absorption system of LRSC when carrying out conventional laser-excited confocal fluorescence microscopy, and to be cognizant of the photochemical complications which could

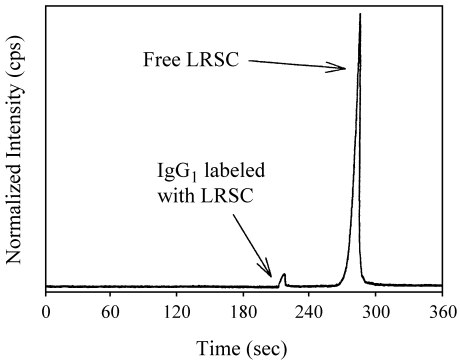

Fig. 4. Relative fluorescence intensity vs. time after injection showing the separation of the LRSC–IgG_1 conjugate from free LRSC in a 10 mM borate buffer at pH = 9.1 using capillary electrophoresis.

result when carrying out two-photon excitation in the red and near infrared regions of the spectrum.

Several reports in the existing literature which describe the photophysical properties of LRSC as a fluorescent probe can now be re-interpreted. First, an early report [9] that LRSC–immunoglobulin (IgG) conjugates become weakly fluorescent and have subnanosecond excited state lifetimes at high dye concentrations is at odds with later reports [6,7] that the fluorescence spectra and lifetimes of free LRSC and of IgG–LRSC conjugates are in the 2–3 ns range and are quite similar. Our observation of a lack of self-quenching or aggregation at LRSC at concentrations up to 0.1 mM support the latter work and suggest that the earlier report is probably incorrect. The previous observation that the fluorescence decays of both free LRSC and IgG–LRSC conjugates are non-exponential [6] is at odds with the present observation of single exponential decays of free LRSC under a wide range of conditions. The difference can be probably attributed to a failure to correct for fluorescence depolarization in the previous studies. The faster of the two components reported in the previous decays can be attributed to rotational depolarization; we have noted the same effect when we do not employ a magic angle polarizer in the emission beam.

The spectroscopic and photophysical data obtained in the present work may be used, together with previous reports of the spectra and fluorescence lifetimes of LRSC-labelled IgG, to obtain a quantitative estimate of the average number of molecules of LRSC bound per IgG species in the dye conjugation reaction. Fig. 4 shows the separation of free LRSC and LRSC bound to human IgG_1 achieved by capillary electrophoresis. Both the spectrum of the IgG–LRSC conjugate and its excited state lifetime ($\tau = 1.86$ ns [7]) are very similar to those measured here for free LRSC under the same conditions. Thus a measurement of the fluorescence intensity of free LRSC relative to that of the LRSC–IgG conjugate may be used to estimate the relative numbers of LRSC fluorophores in the unbound and bound states. The reaction of a large (33:1) molar excess of LRSC with IgG produced the equilibrium distribution of bound and unbound LRSC in the CE separation shown in Fig. 4. Knowing the initial concentrations of LRSC and IgG in the reaction mixture and measuring the relative intensities (peak areas) of the two fluorescence signals in Fig. 4, one obtains [bound LRSC]:[IgG] = 1.9 ± 0.2, or approximately two molecules of LRSC taken up per molecule of IgG under the reaction conditions normally employed for these conjugations. This is in reasonable agreement with a previous estimate of 2–4 conjugated dye molecules per IgG_1 from a study of the labelling of IgG with several different rhodamine dyes [7].

4. Conclusions

Measurements of the spectra and photophysics of LRSC have revealed that a single chemical species, the zwitterion, is responsible for both absorption and emission under circumstances in which the dye is used as a fluorescence probe. The zwitterion's excited state decay constants have been calculated. These results and existing literature data have been used to provide recommendations for the use of this dye when it is being used for quantitative imaging and fluorescence labelling studies. A capillary electrophoresis system with a fibre optic-based fluorescence detector has been employed to separate LRSC and its conjugate with IgG. With quantitative photophysical data in hand, the measured relative fluorescence intensities of the free LRSC and the LRSC–IgG conjugate may be used to determine the stoichiometry of the IgG protein–LRSC dye conjugation reaction. The method should be general for any LRSC–protein or LRSC–phospholipid conjugation reaction, provided separation of the product from the reactants can be easily achieved.

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