Fluorophore spectroscopy in aqueous glycerol solution: the interactions of glycerol with the fluorophore

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Received: 20 July 2021 / Accepted: 24 August 2021 / Published online: 5 October 2021

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Abstract

A common perception exists that glycerol provides an inert-like environment modifying viscosity and index of refraction by its various concentrations in aqueous solution. Said perception is herein challenged by investigating the effects of the glycerol environment on the spectroscopic properties of fluorescein, as a representative fluorophore, using steady-state and time-resolved techniques and computational chemistry. Results strongly suggest that the fluorescence quantum yield, measured fluorescence lifetime (FLT), natural lifetime and calculated fluorescence lifetime are all highly sensitive to the presence of glycerol. Glycerol was found to impact both the ground and first excited states of fluorescein, quenching and modifying both absorption and emission spectra, affecting the fundamental electrical dipoles of the ground and first excited singlet states, and lowering FLT and quantum yield. Furthermore, the Stern–Volmer, Lippert–Mataga, Perrin and Strickler–Berg relations indicate that glycerol acts upon fluorescein in aqueous solution as a quencher and alters the fluorescein geometry. Predictions made by computational chemistry impressively correspond to experimental results, both indicating changes in the properties of fluorescein at around 35% v/v aqueous glycerol, a clear indication that glycerol is not an innocent medium. This study proposes the Strickler–Berg relation as a means of detecting non-negligible effects of a hosting medium on its host fluorophore. These new insights on the molecular structures, the interactions between glycerol and its host fluorophore, and the effects of one on the other may be essential for understanding fundamental phenomena in chemistry and related fields.

Graphic abstract

1 Introduction

The importance of the simple polyol glycerol in cell preservation [1, 2], pharmaceutical formulations [3], investigation of diffusion processes [4] and spectro-photo-fluorescopy [5] is obvious and needs no special justification. However, in the context of the wide field of molecular spectro-photo-fluorescopy, there is a common perception, held by both system manufacturers and users, that glycerol is an inert-like phantom environment, easily approachable, which provides viscosity (from van der Waals interactions and hydrogen bonds [6, 7]) and index of refraction by varying its concentration in aqueous solution. Nonetheless, there are additional specific influences of an aqueous glycerol solution on fluorophores that are usually overlooked [8–12]. For instance, references [13–16] are highly informative regarding fluorescein in various solvents, although they do not consider fluorescein dissolved in aqueous glycerol. This in itself strengthens the argument that many users of glycerol in optophotospectroscopic measurements understand glycerol to be an innocent media. Furthermore, to the best of our knowledge, the interaction at the molecular level between glycerol and fluorophores has not been explored—neither experimentally nor theoretically.

This, in itself, is quite surprising since there are several publications that demonstrate inconsistent behaviour of fluorophores in aqueous glycerol—seemingly enough to justify an inquiry into fluorophore–glycerol interactions. For instance, in one study the measured fluorescence lifetime (FLT) of Rhodamine B was found to be independent of glycerol concentration [8], while other studies found that its FLT is viscosity dependent [17]. In addition, an inverse relation between fluorescein FLT and glycerol concentration has been measured [11, 18]. Furthermore, the FLT of the enhanced green fluorescent protein (EGFP) has been demonstrated to be dependent on the refraction index of aqueous glycerol solutions, but independent of the viscosity [9].

The main aim of this study is to investigate the specific influences of glycerol using fluorescein as a representative fluorophore. Fluorescein is an established fluorophore commonly used in numerous fields of research and applications [19]. Its high fluorescence quantum yield makes for easy detection even at low concentrations, a fact which makes it a standard fluorescence indicator in molecular physics [20], chemistry [19, 20], biology [19, 21] and medicine [22, 23].

The present study utilizes steady-state and time-resolved fluorescence techniques and analyses the results using relations such as Stern–Volmer [24], Lippert–Mataga [25, 26], Perrin [27] and Strickler–Berg [28]. Finally, the theoretical aspects of fluorescein–glycerol interactions are examined. Using density functional theory (DFT), various models of fluorescein–glycerol were constructed and the effects of glycerol coordination

on the geometries and spectroscopic properties of fluorescein were estimated and found to agree with the experimental results.

2 Experimental

Full details are provided in the Electronic Supplementary Information (ESI).

2.1 Materials and methods

2.1.1 Materials

Fluorescein (Sigma-Aldrich, USA) was dissolved in glycerol (Sigma-Aldrich, USA) and double distilled water (0–100% v/v). Additional solvents used are listed in SI along with their properties. All solutions were degassed, with $\text{pH} = 7.3 \pm 0.2$ and fluorescein concentrations of $2 \mu\text{M}$; this yields an optical density (OD) < 0.05 , which prevents any inner filter effects.

2.1.2 Methods

Glycerol was purchased from Sigma-Aldrich and is 99.5% pure with $\text{pH} = 7$ at 25°C . When measuring the pH of glycerol–water solutions, one should be very conscious of the fact that pure glycerol is a very weak acid, that is to say that the concentration of H^+ is very low and, in pure glycerol, it cannot be stabilized by hydration. Furthermore, in practice pH depends on the level of impurities, including the levels of water and dissolved carbon dioxide. Thus, to stabilize the measured pH values, degassed double-distilled water was used.

The following limitations were taken into account when measuring pH , especially of viscous solutions: (a) A hydrated gel outer layer covers the glass bulb of pH electrodes sensing the hydrogen activity (and pH) of the solution. When this hydrated layer is dehydrated or disrupted, the response can be slower and less precise. (b) The aqueous fill solution in the pH electrode may not be miscible with or dissolve in the sample being tested. If this happens, a junction potential may develop and bias the results. (c) The viscosity of the sample may be incompatible with the complex construction of the pH electrode. (d) The sample may have a high impedance resulting in it being a poor electrical conductor.

For this study, an Orion Research Expandable Ion Analyzer EA920 (Orion Research, Inc., Beverly, MA, USA) was used with a resolution of ± 0.002 . The glass electrode was

stored in distilled water. All measurements were performed using buffered solutions.

Before any set of measurements, the pH meter was internally reset and then calibrated at 20 °C using standard buffer solutions: (1) boric acid/potassium chloride/sodium hydroxide—pH 9.00 at 20 °C, and (2) disodium hydrogen phosphate/potassium dihydrogen phosphate—pH 6.8 at 20 °C; both were purchased from Merck KGaA (Darmstadt, Germany) [29]. Next, to ensure complete wetting, the electrode was immersed in the sample at least five times at 22 °C. The sample was constantly mixed with a magnetic stirrer. The measurements were repeated until a stable reading was obtained, washing the electrode between measurements. The final result was taken as the arithmetic average of five readings.

The viscosities of solutions were estimated by a four-parameter correlation function [30] as is common practice [31–33]. The refractive indices of the solutions were measured using a Boeco Abbe refractometer. Absorption spectra were measured by a double-beam Cary 100 spectrophotometer (Agilent, USA). Fluorescence spectra and steady-state polarization were measured by an L shape Cary Eclipse spectrofluorometer (Agilent, USA). In practice, emission intensities are normalized since all excitations were performed at 480 ± 2 nm with 2 μ M fluorescein.

To prevent distortion of fluorescence intensity (FI) measurements by system optics and polarized emission, all FI values in this study are total intensities (I_T), calculated from the fluorescence polarization components as $I_T = I_{\parallel} + 2GI_{\perp}$, [19, 34] where $I_{\parallel}$ and $I_{\perp}$ are, respectively, the fluorescence intensities measured through an analyser polarizer when the plane of polarization is parallel or perpendicular to the electric field vector of the excitation light and G is an instrument-specific correction factor (see Eq. (1) below).

The temperature was controlled using an MTA-48 digital temperature controller (Maxwell, China). Heating of samples was done using a MultiTemp II Thermostatic Circulator (LKB Bromma, Sweden) or with a homemade resistance wire connected to the sample holder.

Measurements of fluorescence lifetimes were performed via both time- and phase-modes by means of an inverted OLYMPUS IX-81 microscope with a 10 $\times$, NA=0.4 objective which was facilitated by (a) a time-domain technique: confocal DCS-120 time-correlated single photon counting, TCSPC (SPC-150, Becker & Hickl GmbH, Berlin, Germany), where the illumination source in the TCSPC was a 471 nm laser diode (Becker & Hickl GmbH, BDL-470-SMC) with a repetition rate of 50 MHz [35], and (b) a frequency domain technique: LIFA-Widefield (Lambert Instruments, Roden, The Netherlands) with a sinusoidal laser source set to a fixed frequency of 20–100 MHz, wavelength

of 468 nm and a CCD camera. All FLTs have a single exponential decay.

For testing the readiness of FLT measurement systems, Rhodamine-123 ($\tau=4.2$ ns) [36], Acridine Orange ($\tau=2$ ns) [37] and tetramethylrhodamine methyl ester (TMRM, $\tau=2.4$ ns) [38] were used as references. The measured values $\tau_M=4.158$, 2.06 and 2.414 ns, respectively, are in good agreement with literature.

2.2 Computational methods

All calculations were done using GAUSSIAN09 Revision E.01 [39] and GAUSSIAN16 Revisions A.03, B.01 and C.01 [40] except for double-hybrid DFT (vide infra) calculations, which were calculated using ORCA versions 3.0.3, 4.1.2 and 4.2.1 [41]. Geometries were optimized using Adamo and Barone's hybrid version (PBE0) [42] of the Perdew–Burke–Ernzerhof (PBE) [43, 44] density functional. Energies were calculated using Martin and coworkers' revised dispersion-corrected (specifically the fourth version of Grimme's dispersion correction—denoted as D4 [45, 46]), spin component scaled (i.e., an SCS-MP2 [47–49]-like correlation contribution), double hybrid (DSD) functional, incorporating the PBE exchange [43, 44] and the Perdew-86 (P86) correlation [50] functionals (i.e., revDSD-PBEP86-D4) [51]. The D4 dispersion corrections were calculated using the code provided by the authors [52]. The D4 dispersion correction, which includes a multibody term, was calculated using the default Axilrod–Teller–Muto (ATM) higher-order dipole–dipole interactions and classical electronegativity equilibration (EEQ) partial charges [46].

For geometry optimizations, the def2-SVP (double- ζ quality) basis set [53, 54] was used while the def2-TZVPP (triple- ζ quality with two sets of polarization functions [53, 54]) basis set was used for the double-hybrid energy calculations.

Bulk solvent effects were approximated using a polarizable continuum model (PCM) [55–58], specifically the integral equation formalism model (IEF-PCM) [55, 56, 59, 60] with water or glycerol as the solvent as in the experiments. Specifically, Truhlar's empirically parameterized version Solvation Model Density (SMD) was used [61]. The SMD model in water, unless stated otherwise, was used for all geometry optimizations and energy calculations (both including TDDFT). Since glycerol is not one of the solvents included in the standard implementation of the SMD model, the solvent-specific parameter (Table S3) were added explicitly.

Excitation energies were calculated using time-dependent DFT (TDDFT) [62–68]. After considering results presented in the literature [69–74], the hybrid-meta version [75] of the Hamprecht–Cohen–Tozer–Handy (HCTH) [76] functional

(τ HCTHhyb) was chosen; this functional had the lowest errors for valence and azabenzene excitation [71] and performed well with respect to Thiel's 28-molecule test set [73] and in a larger benchmark set by Jacquemin [69]. While only the first excitation (root) is of interest, ten roots were included in the TDDFT calculations. Excited state geometries were optimized at the TD-PBE0/def2-SVP level of theory. For both the ground state and excited state optimized geometries, TDDFT energies were calculated using the def2-TZVPP basis set and the SMD solvation model.

For interpretative purposes, Wiberg bond indices [77] and iterative Hirshfeld charges [78, 79] were calculated.

2.3 Theoretical aspect of the measures used

2.3.1 Fluorescence polarization (FP)

Although low concentration fluorescent molecules in solution are distributed isotropically in every sense, the excited molecules are not. So when they are directionally photo-selected by a linearly polarized excitation beam, the right-angle detected fluorescence will be polarized. The fluorescence polarization p is defined as

$$p = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + GI_{\perp}} \quad (1)$$

Generally, the parallel and perpendicular components are distorted by the measurement instrument to different degrees and are corrected by the factor G [19].

The measured fluorescence polarization p of relatively small and spherical molecules [27] hosted in a viscous solution is given by the Perrin equation [27]

$$\left(\frac{1}{p} - \frac{1}{3}\right) = \left(\frac{1}{p_0} - \frac{1}{3}\right) \left(1 + \frac{\tau}{\tau_c}\right) \quad (2)$$

where p_0 is the fundamental fluorescence polarization. When the fluorophore transition dipole moments of the absorption and emission are parallel and the fluorophore behaves as if it were a frozen, noninteracting gas in the hosting medium, then $p_0 \rightarrow \frac{1}{2}$ (see Eq. (15) below) [19]. τ is the FLT and $\tau_c = V\eta/RT$ is the rotational correlation time, where V is the molar hydrodynamic volume $V = 319.1 \text{ cm}^3/\text{mol}$ (see ESI), η is the viscosity of the hosting medium, R is the universal gas constant and T is the absolute temperature. As can be seen from Eq. (2), when $\tau/\tau_c \rightarrow 0$ then $p \rightarrow p_0$, and when $\tau/\tau_c \rightarrow \infty$ then $p \rightarrow 0$, which essentially occurs at very high (100% glycerol) and very low (water) viscosities of the hosting medium, respectively. It can be shown that for randomly-distributed, motionless fluorophores, the fundamental fluorescence polarization is in the range $-1/3 \leq p_0 \leq 1/2$ depending

on the angle between the absorption and the emission transition moments of the fluorophore [20].

2.3.2 Fluorescence quantum yield (ϕ)

The ratio between the number of photons emitted and the number of photons absorbed by a molecule is defined as the fluorescence quantum yield ϕ . For practical reasons, the quantum yield of fluorescein was assessed in this study with respect to the known quantum yield of a reference (r) fluorophore, according to [80]

$$\phi = \phi_r \left(\frac{I}{I_r}\right) \left(\frac{\text{OD}_r}{\text{OD}}\right) \left(\frac{n}{n_r}\right)^2 \quad (3)$$

where I is the total intensity of the emission spectrum, OD is the absorbance at the excitation wavelength, which was kept in this study at ≤ 0.05 , and n is the index of refraction of the solvent at the primary emission wavelength; the subscript r denotes the reference compound. To avoid corrections due to different absorbances, the sample and reference were excited at the wavelength where they have equal absorptions. For determining the ϕ for aqueous glycerol solutions, the published ϕ of fluorescein in water was used as the reference where $\phi_r = 0.92$ [81–83]. The dyes Rhodamine-123 ($\phi = 0.9$) [84], Acridine Orange ($\phi = 0.25$) [85] and TMRM ($\phi = 0.28$) [85] were measured, and the results ($\phi = 0.9, 0.2$ and 0.27 , respectively) are in excellent agreement with literature.

2.3.3 The natural lifetime (τ_0)

The quantum yield ϕ is related to the measured fluorescence lifetime τ and to the natural lifetime τ_0 by [19, 20]

$$\tau = \tau_0 \phi \quad (4)$$

Thus, by measuring τ and ϕ , one can calculate τ_0 . Nonetheless, given that [19]

$$\tau = \frac{1}{K_r + K_{nr}} \quad (5)$$

where K_r and K_{nr} are the rate constants for radiative and nonradiative deexcitation processes, one can use the relation between the natural lifetime and its rate constant ($1/K_r = \tau_0$) and Eq. (4) to obtain

$$\tau = \phi \cdot \tau_0 = \frac{\phi}{K_r} \Rightarrow K_r = \frac{\phi}{\tau} \quad (6)$$

Substituting Eq. (6) into Eq. (5) one gets:

$$\tau = \frac{1}{\phi/\tau + K_{nr}} \Rightarrow K_{nr} = 1/\tau(1 - \phi) \quad (7)$$

Further explanations for other measures used in this study are given in the Sect. 3.

3 Results and discussion

3.1 Effect of quenching on absorption and fluorescence spectra

The absorption and emission spectra of fluorescein in aqueous glycerol solutions (0–70%) were measured at 25 °C. The band pass never exceeded 2 nm. For emission spectra, all excitations were at 480 ± 2 nm. All absorption intensities can be considered as absorbances since they were all performed at the same fluorescein concentration of 2 μ M (Fig. 1). The results shown in Figs. 1 and S3 (ESI) show similar behaviours, even though they were obtained at 25 and 37 °C, respectively, indicating negligible, if any, temperature dependence in this range.

A close look at Fig. 1 reveals several interesting features. As the concentration of glycerol increases, the absorption and emission spectra are more redshifted and become less intense. This suggests that glycerol influences both processes, namely it affects both the ground and excited states of fluorescein. Furthermore, normalizing the curves of Fig. 1 (each curve with respect to its maximum – see Figure S2) reveals that with increasing glycerol concentration the absorption curves are redshifted by about 5 nm and the full width at half maximum (FWHM) of the absorption spectra increases from 34 nm in water to 66 nm in 70% glycerol while the emission spectra FWHM increases from 39 to 66 nm towards the longer wavelengths. In addition, with increasing glycerol concentration, the absorption and emission spectra become less similar, and the mirror of symmetry between absorption and emission spectra decreases (see ESI).

Since the reduction of the fluorescence was observed in the absence of a defined quencher molecule, and cannot be justified by temperature-induced collisional deexcitation in this temperature range, the possibility, though uncommon, that glycerol alone could act as a quencher upon fluorescein was examined using the Stern–Volmer relation.

There are two main quenching processes. Dynamic quenching mainly occurs due to collisions between the quencher and fluorophore when the latter is in its excited state S_1 . Dynamic quenching, therefore, acts as an additional channel for radiationless deexcitation thereby shortening FLT and decreasing the quantum yield ϕ . Increasing temperature enhances diffusion and, consequently, collision rates. In dynamic quenching, the fluorophore does not change chemically. Static quenching occurs while the molecule is in its ground state S_0 . The quencher usually chemically acts upon the fluorophore, and as a consequence might reduce its ability to fluoresce. Since static quenching is not related to the excited states, it does not

depend upon the diffusion of the quencher nor on the temperature, unless weak bonds within the fluorophore–complex (which cause the fluorophore to fluoresce) break as the temperature increases thereby reducing the number of fluorophores.

These processes, separately and together, are described by the Stern–Volmer relation [24]

$$\frac{F_0}{F} = 1 + k_q \tau_0^{SV} [Q] = 1 + K_D [Q] \quad (8)$$

where F_0 and F are the steady state peak fluorescence intensities in the absence and presence of the quencher, respectively, which within the context of our work are aqueous glycerol and water, k_q is the intermolecular quenching coefficient, τ_0^{SV} is the FLT of the fluorophore in water alone (i.e., in the absence of a quencher), $[Q]$ is the concentration of the quencher (i.e., glycerol) and K_D is the dynamic quenching rate. When the quenching is purely dynamic (i.e., the source of quenching is solely collisions), then [19]

$$\frac{\tau_0^{SV}}{\tau} = 1 + k_q \tau_0^{SV} [Q] = 1 + K_D [Q] \quad (9)$$

and, following Eq. (8),

$$\frac{F_0}{F} = \frac{\tau_0^{SV}}{\tau} \quad (10)$$

where τ and τ_0^{SV} are the measured FLT values in the presence and absence of the quencher. However, it can be shown that when both dynamic and static quenching occur, the linear relation in Eqs. (8)–(10) becomes a second-order dependency (second order Stern–Volmer equation[24]):

$$\frac{F_0}{F} = 1 + (K_D + K_S)[Q] + K_D K_S [Q]^2 \quad (11)$$

The ratios F_0/F (upper curve) and τ_0^{SV}/τ (lower curve) are plotted in Fig. 2. For both temperatures, the curves clearly show parabolic dependence of the intensity ratios with glycerol concentration, which supports the supposition that glycerol might interfere with both the ground and excited states of fluorescein.

Even though static quenching applies only to the ground state, it nonetheless affects the dynamic quenching by reducing the quantity of ground state molecules, consequently reducing the quantity of excited fluorophores (i.e., the emission intensity). Despite the decreased fluorescence intensity, its lifetime (τ_0^{SV}) remains almost unaffected. For instance, as can be seen from the static quenching shown in Fig. 2, the decrease in the intensity of the fluorescence is about 600–700% while the relation τ_0^{SV}/τ grows negligibly, altogether by 10% over the same range of glycerol concentrations (0–70%), at both 25 and 37 °C. This latter change could be related to noise. Alternatively, since it

Fig. 1 Absorption (solid lines, left axis) and emission (dashed lines, right axis) spectra of fluorescein in aqueous glycerol measured at 25 °C at various glycerol concentrations. The solid vertical lines are added for visual reference only

is a linear and continuous change, it could be attributed to traces of quenchers or other impurities in the glycerol. Nevertheless, if one views this change as real and significant, then the possibility of real dynamic quenching should be considered, and the dynamical quenching constant can be estimated from the slope [19].

Summarizing Fig. 2, the parabolic curvature of F_0/F and the mild linear dependence of τ_0^{SV}/τ suggest that static dilution of fluorescein in its ground state occurs in the presence of glycerol (*i.e.*, the ground state of fluorescein is partially diluted by glycerol, probably in agreement with reference [19]). Another point to consider is the linearity of the curves in Fig. 2 below ~35% glycerol (indicated by dashed line in Fig. 2). Although it might be attributed to the low concentrations, a different explanation will be suggested hereinafter in Sect. 3.5.

A similar break in the properties of fluorescein was observed at 40% glycerol by Feldman and coworkers in their differential scanning calorimetry (DSC) experiments [7, 86]. Likewise, a similar break was found at around 35% for Rhodamine 123 in aqueous glycerol (Figure S4). The dependence of the absorption and emission spectra on glycerol concentration is likely fluorophore-dependent.

It should be noted that the Stern–Volmer analysis is typically applied in situations where the fluorescence quencher is in the micro- to millimolar concentration

range in which there is dynamic quenching due to diffusion. Here, the quencher is glycerol, which is at much higher concentrations (see Table S2). Nevertheless, this analysis is included here as further support of the proposed hypothesis that with increasing glycerol concentration the glycerol takes an active role in affecting the spectroscopic properties of chromophores in general and of fluorescein

Fig. 2 The dependence of F_0/F (left axis) at 25 °C (solid blue circles) and at 37 °C (solid red circles) and τ_0^{SV}/τ (right axis) on glycerol concentration at 25 °C (green triangles) and at 37 °C (purple squares). The dashed vertical line marks the concentration at which the behaviour of the curves change (see text)

in particular. As will be discussed in the DFT study below (Sect. 3.5), glycerol forms complexes with fluorescein thereby affecting its absorption and emission properties. The first glycerol coordinates to the carboxylic acid moiety already at low glycerol concentrations. At higher concentrations, glycerol starts binding to the xanthene fragment (see Scheme 1). Since the latter will have the larger effect on the spectroscopic properties, it is only at higher concentrations, not the millimolar levels, that the effects of quenching are observed. It should also be noted that Eq. (11) is usually applied to fluorophore–quencher dimer aggregates. Here, as noted, one could view the fluorescein–glycerol dimer as the fluorophore and the second glycerol as the quencher. Regardless, this is just one piece of supporting evidence that glycerol is not as innocent as has been often thought.

3.2 Effect of glycerol on the fluorescein absorption and emission transition moment dipoles

The Lippert–Mataga equation (LM) relates the difference between the wavenumbers of the maxima of absorption and emission curves (spectra shift, $\Delta\bar{\nu} = \bar{\nu}_A - \bar{\nu}_F$) of a fluorophore solute and the macroscopic polarizability of the host, ignoring any possible intermolecular (glycerol–fluorescein) interactions [25,26,87]:

$$\Delta\bar{\nu} = \bar{\nu}_A - \bar{\nu}_F = \frac{2}{hca^3} \Delta f (\mu_E - \mu_G)^2 + \text{constant} \\ = \frac{2}{hca^3} \Delta f (\Delta\mu)^2 + \text{constant} \quad (12)$$

$$\Delta f \equiv \frac{\epsilon_r - 1}{2\epsilon_r + 1} - \frac{n^2 - 1}{2n^2 + 1} \quad (13)$$

where Δf is the orientation polarizability, μ_G and μ_E are the fundamental dipole moments of the ground and excited states and $\Delta\mu$ is their difference, a is the Onsager cavity radius [19], and ϵ_r and n are the dielectric constant and refractive index of the host (aqueous glycerol).

It has been demonstrated that using the Lippert–Mataga equation to analyse the solvatochromic properties of a sample known to exhibit solute–solvent interactions could be problematic, such as in the recent study of fluorescein in different protic solvents [16]. This should not be an issue here since the physical properties, such as pH and temperature, that might affect the solvatochromism of glycerol are kept constant between measurements and only the glycerol concentration is adjusted.

The absorption and emission spectra in Fig. 1 are largely invariant with $\Delta\bar{\nu} = 907 \text{ cm}^{-1}$ for all glycerol concentrations. The Cary Eclipse instrument has a minimum resolution

Scheme 1 Possible binding sites. Fluorescein (left) and glycerol (right). Arrows a–d indicate the possible sites on fluorescein for glycerol coordination via its terminal (t) and internal (i) positions. The xanthene fragment of fluorescein is shown in blue

of 1.5 nm, and since this $\Delta\bar{\nu}$ corresponds to a wavelength difference $\Delta\lambda = \lambda_A - \lambda_F \cong 25 \text{ nm}$, this is well within its capabilities. Regarding the calculations done via Eq. (12), the number of interest is the wavenumber of the maxima of each curve. The wavenumbers $\bar{\nu}$ needed for Eq. (12) were extracted from the spectra in Fig. 1 using the built-in software in the Cary Eclipse spectrometer. Each spectrum was measured at least three times, with excellent repeatability between measurements, from which the difference $\Delta\bar{\nu} = \bar{\nu}_A - \bar{\nu}_F$ could be determined.

The dependencies of $\Delta\bar{\nu}$ and $(\Delta f)^{-1}$ on glycerol concentration are depicted in Fig. 3. Below a glycerol concentration of ~30%, $\Delta\bar{\nu}$ remains fairly constant and thereafter decreases only slightly. This behaviour of $\Delta\bar{\nu}$, in a way, forces the right side of Eq. (12) to also remain constant. The practical meaning of this observation is evident when Eq. (12) and (13) are reorganized by moving the constant from the end of the right side of Eq. (12) to the left and multiplying both sides by $\frac{1}{2}hca^3$ yielding

$$\text{constant} = \left(\frac{\epsilon_r - 1}{2\epsilon_r + 1} - \frac{n^2 - 1}{2n^2 + 1} \right) \cdot (\mu_E - \mu_G)^2 = \Delta f \cdot (\Delta\mu)^2 \quad (14)$$

Since changing the glycerol concentration also changes both ϵ_r and n —and consequently Δf —it follows from Eq. (14) that $\Delta\mu$ must change correspondingly so that the product $\Delta f \cdot (\Delta\mu)^2$ remains constant for all glycerol concentrations. This conclusion hints at the interaction of glycerol with fluorescein at the molecular level affecting the fundamental electrical dipole of the ground state, the first excited singlet state or both. Another observation from Fig. 3 is that the behaviour of both $\Delta\bar{\nu}$ and $(\Delta f)^{-1}$ changes at around 35–40% glycerol concentration (dashed line), similar to what is observed in Fig. 2.

The Perrin equation (Eq. (2)) is another indicator of changes in the fluorescein dipole moment in aqueous glycerol solution [27]. Here, the fundamental polarization (p_0)

is extracted for each glycerol concentration (i.e., for each η , τ and τ_c), and the results are plotted in Fig. 4. As the concentration of glycerol increases, p_0 decreases further from the maximum value of 0.5. Other instances where $p_0 = 0.5$ cannot be reached are (a) perylene, possibly due to it being slightly twisted in the ground state [88], and (b) xanthene dyes, due to inner molecular folding and their butterfly-like shape [88].

The angle α between the intramolecular $S_0 \rightarrow S_1$ absorption and $S_1 \rightarrow S_0$ emission transition dipole moments is related to the fundamental fluorescence polarization p_0 by:

$$p_0 = \frac{3 \cos^2 \alpha - 1}{\cos^2 \alpha + 3} \quad (15)$$

Rearranging this equation gives the angle α as

$$\alpha = \cos^{-1} \left(\sqrt{\frac{1 + 3p_0}{3 - p_0}} \right) \quad (16)$$

From the results in Fig. 4, the higher the glycerol concentration, the lower p_0 and consequently, according to Eqs. (15) and (16), the larger the angle α is. Furthermore, from Eq. (15) at 70% glycerol, α reaches 30° , which might

indicate a non-negligible distortion of the molecular geometry, in agreement with previous observations [18].

The Lippert–Mataga equation and measurements of fundamental polarization indicate, with a high level of certainty, that aqueous glycerol solution is not an inert environment but rather one that non-negligibly impacts the spectroscopic and structural features of the hosted fluorophore.

3.3 The dependency of τ and ϕ on various environmental conditions

To examine the effect of a number of environmental variables, specifically viscosity (η), temperature and dielectric constant, on the fluorescein lifetime and quantum yield, τ and ϕ were plotted against the viscosity and index of refraction in different hosting media. By changing the temperature one can adjust the viscosity of the solution. Thus, one can examine the influence of a single factor, such as viscosity, on τ even though different solvents are used.

To examine the effects of various environmental parameters, a selection of solutions was used: aqueous solutions of glycerol (0–70% at 25, 30 and 37 °C), sorbitol (0–50% at 20 °C), sucrose (0–50% at 20 °C), ethanol (0–40% at 20 °C)

Fig. 3 The dependencies of $\Delta\bar{\nu}$ (top) and $(\Delta f)^{-1}$ (bottom) on glycerol concentrations at 25 °C. The dashed vertical lines show the approximate point where the behaviours of $\Delta\bar{\nu}$ and $(\Delta f)^{-1}$ change. The two solid lines in each figure emphasize the different slopes of each part of the plot and were fit using linear regression

Fig. 4 Dependence of the fundamental polarization p_0 (top) and the angle α (in °) between the intramolecular $S_0 \rightarrow S_1$ absorption and $S_1 \rightarrow S_0$ emission transition dipole moments (bottom) on glycerol concentration measured at 25 °C (blue) and 37 °C (red)

and dimethylsulphoxide (DMSO, 0–100% at 25 °C). The first three sets of solutions were chosen to determine the dependency of τ on viscosity. Using ethanol solutions provides insight on hydrogen bonding, while DMSO provides insight into the effects of varying the dielectric constant.

The dependence of τ on viscosity is depicted in Fig. 5. As one can see, there is an inverse relation between η and τ . Moreover, in the enlargement in the lower panel of Fig. 5, the curves overlap, indicating that, at least until 5 cP, the behaviour of τ is independent of the solvent properties other than viscosity. The selection of media used provides a large range of solvent properties, including dielectric constant and hydrogen-bonding ability. It seems that of the various environmental parameters, viscosity is the dominant factor affecting fluorescence lifetimes of fluorescein, while the others only have minor influences. However, one significant hidden variable in Fig. 5 is the glycerol concentration, which increases with viscosity and might weaken the argument that the decrease in τ is solely due to viscosity. The possible influence of glycerol concentration is discussed hereinafter.

Since for all practical purposes there is an overlap of the τ versus viscosity curves at 25 °C and 37 °C (Fig. 6), this suggests that for fluorescein τ is largely independent of the temperature, at least within this range. One, however, might expect increased collisional deexcitation, which would result in reduced lifetimes and quantum yields [20]. Likewise, one can expect deformation of the fluorescein geometry, deduced from the associated changes in the fundamental fluorescence polarization, as shown in the previous section (Fig. 4).

The viscosity of glycerol is exponentially temperature-dependent [89], yet in practice, while this might be counterintuitive to some extent, its concentration barely changes between 25 and 37 °C [90]. The dependence shown in Fig. 6 of τ on the glycerol concentration at these two temperatures strongly suggests, despite the exponential dependence of viscosity, that the influence of temperature-induced viscosity changes on τ is fairly negligible compared to the influence of the glycerol concentration. Having noted that other factors, including viscosity, dielectric constant and hydrogen donating ability, do not have major impacts on τ , it would appear that it is glycerol that has the primary impact on the fluorescein FLT rather than temperature, and this is further emphasized by the FLT of fluorescein obtained in different glycerol concentrations and temperatures (Table 1). Even though there are large changes in temperature and viscosity, for a given glycerol concentration there is little variation in τ . On the other hand, the higher the glycerol concentration, the shorter the fluorescein FLT. This suggests that a direct glycerol–fluorophore interaction is a likely contributor to the shortened FLT.

The dependence of FLT on the index of refraction (n) is shown in Figure S5. There is a clear inverse relation between

Fig. 5 Dependence of the fluorescein FLT (τ , ns) on the viscosity in various aqueous solutions: 0–70% glycerol, 0–50% sucrose, 0–60% sorbitol, 0–40% ethanol and 0–100% DMSO. Upper panel: entire viscosity range. Lower panel: magnification of the 0–5 cP range. Temperatures and solvents are defined in the legend in the upper panel

FLT and n , and with the exception of DMSO, which has special properties *vide infra*, there is a similar behaviour for all solutions.

The viscosity and density of DMSO in water have a unique behaviour. As observed by LeBel and Goring, the viscosity and density of aqueous DMSO solutions increase to a maximum of 3.8 cP and 1.10 g/mL at a concentration of ~67% DMSO after which they both decrease [91]. For fluorescein, the FLT decreases with increasing DMSO concentration (density) and is not affected by the solution viscosity (Figure S6). Therefore, clearly the FLT is not affected by the solution viscosity in this case.

The dependence of the quantum yield ϕ (Eq. (3)) on glycerol concentration and temperature is presented in Fig. 7. As expected, higher temperatures increase the chance for nonradiative collisional deexcitation, consequently decreasing ϕ (and τ) [20]. Thus, the 25 °C curve is above the 37 °C curve for all glycerol concentrations. However, with elevated glycerol concentration, one would have expected the glycerol viscosity to act against the collision dynamics [92, 93] and hence increase ϕ (and τ), even though glycerol viscosity exponentially decreases with temperature. Nonetheless, the fact that the different temperature curves converge at high glycerol concentrations suggests that collisional dynamics

Fig. 6 The dependence of fluorescein τ on glycerol concentration (0–70% glycerol in water) and temperature (25 °C—blue, 37 °C—red). Coefficient of variance (CV) values never exceeds 5%

Table 1 FLTs (τ , ns) and viscosities (η , cP) in aqueous glycerol solutions with different concentrations and temperatures (T , °C)

T	49% glycerol		70% glycerol	
	η	τ	η	τ
25	4.756	3.775	18.057	3.651
30	4.024	3.764	14.368	3.639
37	3.244	3.794	10.711	3.682

is indeed slowed down by glycerol viscosity, though less dominantly (in this temperature range) than the quenching of fluorescein by glycerol. Moreover, since these experiments were performed in the absence of an added quencher, the decrease of ~50% in ϕ further supports the conclusion that glycerol acts as a quencher at the level of glycerol–fluorescein intermolecular interactions.

It is also interesting to note that the slopes of the curves in Fig. 7, especially for 37 °C, seem to change quite significantly at ~35% glycerol (see dashed vertical line), resembling the behaviour observed in Fig. 2 and Fig. 3. This will be discussed later.

The complementary aspect of the above discussion would be the rate constant for nonradiative deexcitation (k_{nr}) calculated using Eq. (7) (Table 2). The higher the glycerol concentration, the larger k_{nr} , indicating the action of an apparent quencher that does not obey the common postulate that increasing viscosity should slow down the dynamics of molecular quenchers [19] and consequently reduce k_{nr} . In principle, one could extract K_D and K_S from a series of time-resolved and steady-state experiments using Eqs. (9) and (11), but this is beyond the scope of the present study.

To further emphasize the dominance of glycerol over temperature in quenching fluorescein, the results of ϕ obtained in water and in aqueous glycerol at three temperatures are

Fig. 7 The dependence of the quantum yield ϕ of fluorescein on glycerol concentration in water (25 °C—blue, 37 °C—red)

listed in Table 3. It can be observed that ϕ decreases faster in water than in aqueous glycerol. On the other hand, at a given temperature, there is a dramatic attenuation of ϕ by glycerol.

Closely comparing Figs. 1 and 7 shows that both the fluorescence intensity peak and fluorescence quantum yield have similar inverse dependencies on glycerol concentration. However, these curves do not have the same slopes, which is unusual, yet it would be reasonable for there to be a change in spectral properties, including fluorescence intensity and quantum yield, according to the proposed hypothesis, presented in more detail in the DFT study below, that there is a change in the coordination sphere of fluorescein upon increasing glycerol concentration. In the present study, other peculiar behaviours were noted. For instance, for fluorescein, both the absorption and emission intensities are attenuated with glycerol concentration (Figs. 2 and S4). In contrast, for Rhodamine B both are intensified while for Rhodamine 123, the absorption increases with increasing glycerol concentration while the emission is almost independent of glycerol concentration (Figure S7). These inconsistent behaviours, which are the subject of ongoing studies, further question the perception of glycerol as an inert medium.

3.4 Effect of glycerol on fluorescein using Strickler–Berg equation

In addition to Eq. (4) for estimating τ_0 , in several cases τ_0 may be estimated using the Strickler–Berg (SB) relation [28]:

$$\frac{1}{\tau_{0, \text{cSB}}} = 2.88 \times 10^{-9} \cdot n^2 \cdot \frac{\int I(\tilde{\nu}) d\tilde{\nu}}{\int \tilde{\nu}^{-3} I(\tilde{\nu}) d\tilde{\nu}} \cdot \int \frac{\epsilon(\tilde{\nu})}{\tilde{\nu}} d\tilde{\nu} \quad (17)$$

$$\approx 2.88 \times 10^{-9} \cdot n^2 \cdot F \cdot A$$

where n is the index of refraction of the hosting solvent at the maximum emission wavelength of the fluorophore, $I(\tilde{\nu})$ is the emission intensity, $\epsilon(\tilde{\nu})$ is the molar absorption coefficient, $\tilde{\nu}$ is the wavenumber (in cm^{-1}) of the emission, and

Table 2 Nonradiative deexcitation rate constant (k_{nr} , /ns), fluorescence quantum yield (ϕ , unitless) and FLT (τ , ns) of fluorescein in various aqueous glycerol concentrations measured at 25 °C

% Glycerol	τ	ϕ	k_{nr}
0	4.018	0.927	0.018
12	3.94	0.915	0.021
23	3.895	0.89	0.028
34	3.852	0.82	0.046
42	3.811	0.789	0.055
49	3.775	0.75	0.066
63	3.698	0.67	0.089
70	3.65	0.58	0.115

Table 3 Quantum yield (ϕ) of fluorescein in water and 49% and 70% glycerol at different temperatures (T , °C)

T	Water (0% glycerol)	49% glycerol	70% glycerol
25	0.92 [81]	0.75	0.58
30	0.872	0.734	0.577
37	0.828	0.726	0.573

F and A are the fluorescence ratio integral and the absorption integral over the emission and absorption spectra of a fluorophore, respectively. However, Eq. (17) does not apply to situations where the fluorophore (a) interacts with the hosting media, (b) is built of substituted aromatic components or behaves similarly, (c) is quenched to a degree that cannot be ignored, (d) has a relatively weak (i.e., $\epsilon_{\max} < 8000 \text{ M}^{-1}\text{cm}^{-1}$) $S_0 \rightarrow S_1$ transition, or (e) has a first excited singlet state that is non-negligibly deformed [19].

From our experience, these principal limitations do not hold for fluorescein. Fluorescein does not have a weak $S_0 \rightarrow S_1$ transition ($\epsilon_{\max}^{0\% \text{ glycerol}} \approx 74,257 \text{ M}^{-1}\text{cm}^{-1}$, [94] $\epsilon_{\max}^{70\% \text{ glycerol}} \approx 13,979 \text{ M}^{-1}\text{cm}^{-1}$), and hence the use of the SB equation for fluorescein in water should probably yield a reasonable value for the natural lifetime $\tau_{0,\text{CSB}}$. Using Eq. (4), one can estimate the measured lifetime τ from the calculated $\tau_{0,\text{CSB}}$ and the measured ϕ .

Because of the abovementioned limitations of SB formula, we suggest it as a means for identifying deviations of fluorophores from the “eligible SB state.” In this study, we make use of the SB relation to discern at which glycerol concentration (in water) the behaviour of fluorescein stops obeying the SB relation. In particular, we define deviations of 25% between $\tau_{0,\text{CSB}}$ and τ_0 to be the point at which the fluorophore no longer follows the SB relation due to environmental influences. The contributions of each component of $\tau_{0,\text{CSB}}$ is listed in Table S4.

The plots of $\tau_{0,\text{CSB}}$ and τ_0 versus glycerol concentration are shown in Fig. 8. The two plots have identical trends, but starting from about 35% glycerol the separation between these two curves becomes significant, a familiar

Fig. 8 Dependence of $\tau_{0,\text{CSB}}$ (red, calculated using Eq. (16)) and τ_0 determined from direct measurements (blue) of fluorescein in various concentrations of glycerol in water

phenomenon already observed for other comparisons. Around this glycerol concentration, the hosting medium alters the hosted fluorophore features so that it no longer complies with the limitations of the SB relation. It is interesting to note that, although they did not use aqueous glycerol solutions, fluorescein was one of the fluorophores used by Strickler and Berg to experimentally demonstrate the validity of their formula [28].

3.5 Understanding glycerol coordination using DFT

3.5.1 General

All calculations were done using DFT at the SMD-revDSD-PBEP86-D4/def2-TZVPP//SMD(H_2O)-PBE0_{D3BJ}/def2-SVP level of theory; see Sect. 2.1.3 for full details. Gibbs free energies herein are for a standard state of 298.15 K (25 °C) and 1 atm; the free energies can be corrected for the various glycerol concentrations as discussed in the ESI. In order to model the bulk solvent effects in a mixture of two solvents (i.e., water and glycerol), any property (e.g., energy, absorption and emission wavelengths and oscillator strengths) will be approximated as the weighted average of the property in each neat solvent using the solvent fraction as the weight. Thus, for any property $P(n)$ in $n\%$ glycerol:

$$P(n) = \frac{n}{100}P(\text{glycerol}) + \frac{100-n}{100}P(\text{water})$$

It is reasonable to assume that these properties will change monotonically as one moves from one solvent to the other. Water is available as a solvent in standard implementations of the SMD model, while the parameters for glycerol were taken from the literature (Table S3).

The pKa of glycerol is 14.15 [95] and will thus always remain protonated. However, the phenolic pKa of fluorescein (i.e., pKa₂) is 6.35 [96] and therefore, at the experimental

pH of 7.3 ± 0.2 used in this study, it will always be doubly deprotonated.¹

Fluorescein (FS) has five binding sites—four unique—while glycerol (G) has three binding sites—two unique (Scheme 1). This allows for various bonding combinations, especially as more than one molecule of G can bind to a single FS. The nomenclature used herein for FS:G complexes identifies the sites on FS and on G—the latter as a subscript—involved in the intermolecular interaction. For example, a_i would be coordination of the glycerol internal OH to site A (one of the two carboxylic oxygen atoms closer to the xanthene fragment), $a_i b_i$ would be coordination of the internal OH to one carboxylic oxygen and one of the two terminal OHs to the other carboxylic oxygen, and $c_i c_i$ would be coordination of both glycerol terminal OHs to the ring O—. Glycerol is most likely too small for one glycerol to bind to two fluorescein sites, with the exception of sites A and B (the two oxygens of the carboxylate group). In all cases, while not explicitly noted in these labels, the dianion FS^{2-} is implied.

3.5.2 Geometry of fluorescein

The structure of FS^{2-} is shown in Fig. 9A. One key feature is that the phenyl ring is nearly perpendicular to the xanthene ring (the dihedral angle δ (C8–C7–C14–C15) = 74.3° —see Table 4 upper panel for atom numbering scheme). The phenyl ring cannot be coplanar, as might be expected from aromaticity considerations, because of steric repulsion between the carboxylate group and the C–H moiety (C13–H or C2–H). The potential energy surface for rotation of the phenyl ring with respect to the xanthene moiety is fairly flat around perpendicularity ($\pm 50^\circ$ rotation raises the energy by no more than 4.0 kcal/mol, Figure S6).

It has been observed for various properties where there may be solute–solvent interactions, such as reaction energies [97], IR [98], UV–vis [98], Raman optical activity (ROA)

[97], NMR chemical shifts [98–100] and EPR parameters predictions, that including one or more explicit solvent molecules can improve the property predictions [101]. Therefore, in order to obtain more reliable absorption and emission predictions for fluorescein, a complex with four water molecules was optimized (Fig. 9B). While one water molecule was placed on each high-negative charges sites (Table 7), specifically the two carboxylate oxygens and the two xanthene oxygens (*i.e.*, O1, O2, O4 and O5), one water migrated from O4 to form a hydrogen-bridge network.

3.5.3 Geometries of 1:1 fluorescein–glycerol complexes

All 1:1 complexes were considered. The relative energies are listed in Table 5 and selected geometries are shown in Fig. 9. Clearly, the preferred coordination of glycerol is to the carboxylate. The two lowest-energy complexes have the glycerol bound by its internal and one terminal OH groups— $a_i b_i$ and $a_i b_i$. The next in energy is coordination to the ring O1/O2 (c) and then coordination to the bridging O3 (d). The energies in Table 5 are corrected for glycerol concentration using the corrections in Table S2. Even at the lowest concentration used (12% glycerol), the formation of the complexes is feasible given that the glycerol is present in an approximate one million-fold excess. A Boltzmann distribution between free $FS^{2-} \cdot 4H_2O$ /glycerol and all 1:1 complexes (Table 6) predicts 93% complexation at this glycerol concentration, with $a_i b_i$ and $a_i b_i$ accounting for 84% of all fluorescein.

Selected bond distances are listed in Table 4 for two of the complexes. The largest change (in magnitude) upon glycerol coordination is 0.017 Å (C1–C6 in $c_i c_i$). There are also some small changes in the vicinity of the glycerol, notably elongation of the O–H bonds as a result of the hydrogen bonding to fluorescein. These patterns are reflected in the Wiberg bond orders, and the iterative Hirshfeld charges (Table 7) also support a hydrogen-bonded complex with transfer of electron density to the glycerol.

3.5.4 Geometries of 1:2 fluorescein–glycerol complexes

To a number of the lower energy 1:1 complexes, the glycerol-O moieties of the c_i , $c_i c_i$ and $c_i c_i$ complexes were added and the geometries were optimized (Fig. 9). Complexation of the second glycerol is not as favourable as the first (Table S6), and at 12% glycerol only the formation of the lowest energy isomer is feasible, and then only barely with $\Delta G_{\text{complex}}^{12\%} = -0.3$ kcal/mol. At this glycerol concentration, only 20% of the FS^{2-} is coordinated by two glycerol molecules (Table S5) while 75% is complexed with one glycerol and 4% remains free. Because the concentration correction increases with the number of complexed

¹ We are not aware of pKa measurements for fluorescein in non-aqueous solutions and binary solvent mixtures. Thus, we cannot be definite regarding the protonation state of the fluorophore. However, it is reasonable to assume that in solvents with a large proportion of water, the behaviour of the solution will more resemble that of the aqueous solution—and it is at these predominantly aqueous solutions where the spectrochemical behaviour of fluorescein is seen to change. It should also be noted that deprotonation provides a larger conjugated π -system, which is a significant stabilizing effect. Regardless, the hydroxy (–OH) group is still a good hydrogen-bond partner. In principle one could calculate the pKa of fluorescein in all the various solutions. However, calculating pKa is notoriously finicky due to the logarithmic nature of pKa, which will magnify any small errors in the calculation. Three potential sources of error are the inherent errors in the (DFT) calculation method, the unknown solvation properties of the proton in various media, and deviations from the assumed linear monotonicity of the Gibbs free energy between 100% water and 100% glycerol.

Fig. 9 Optimized geometries of selected fluorescein:glycerol complexes: **A** FS^{2-} , **B** $\text{FS}^{2-} \cdot 4\text{H}_2\text{O}$, **C** a_1b_1 , **D** a_1b_1 , **E** $a_1b_1b_1$, **F** c_1c_1 , **G** $a_1b_1b_1 + c_1c_1$, **H** $a_1b_1b_1 + c_1c_1 + c_1c_1$. Colour scheme: C—black, H—white, O—red

glycerol molecules (Table S2), with increasing glycerol concentration the proportion of 1:2 complexes increases, and at 42% glycerol the ratio is 50:49:1, respectively.

3.5.5 Geometries of 1:3 fluorescein–glycerol complexes

Two 1:3 complexes were found (Fig. 9H) by adding a second c_1c_1 moiety to the lowest energy 1:2 complex; two alternate orientations were considered. Based on their complexation energies (Table S6), they are present only at the higher

glycerol concentrations (Table S7). If one plots the distribution of each type of complex (Fig. 10), one notes that there is a crossing of the 1:1 and 1:2 populations. In the low concentration regime (not shown since not considered experimentally), only 1:1 complexes will form with rapid increase from 0% at 0% glycerol, which will then begin the observed decay as 1:2 and, eventually, 1:3 complexes start to form. Fitting each population to a logarithmic function (i.e., $y = a \ln(x) + b$, Fig. 10) shows that these two cross at 40% glycerol with 47% population of each. This crossing is

Table 4 Selected bond lengths (Å) and Mayer bond orders in FS²⁻ and two selected 1:1 complexes with the number scheme shown in the upper panel and the larger changes in bold

Bond	FS ²⁻		<i>a</i> ₁ <i>b</i> _t		<i>c</i> _t <i>c</i> _i	
	<i>d</i> (Å)	Mayer BO	<i>d</i> (Å)	Mayer BO	<i>d</i> (Å)	Mayer BO
C1–C2	1.365	1.64	1.365	1.64	1.371	1.59
C1–C6	1.455	1.14	1.456	1.14	1.438	1.20
C2–C3	1.429	1.19	1.428	1.19	1.421	1.23
C3–C4	1.428	1.17	1.429	1.17	1.420	1.21
C4–C5	1.375	1.51	1.374	1.51	1.382	1.45
C5–C6	1.438	1.20	1.439	1.20	1.422	1.27
C3–C7	1.408	1.31	1.406	1.31	1.418	1.25
C7–C8	1.408	1.30	1.407	1.31	1.399	1.35
C8–C9	1.428	1.18	1.429	1.17	1.432	1.16
C9–C10	1.375	1.51	1.374	1.51	1.373	1.52
C10–C11	1.438	1.20	1.439	1.20	1.439	1.19
C11–C12	1.455	1.14	1.455	1.14	1.458	1.13
C12–C13	1.366	1.64	1.365	1.64	1.362	1.66
C8–C13	1.428	1.20	1.429	1.19	1.431	1.18
C7–C14	1.489	0.99	1.488	0.99	1.490	0.98
C14–C15	1.408	1.35	1.409	1.34	1.407	1.35
C15–C16	1.400	1.39	1.399	1.39	1.400	1.39
C16–C17	1.392	1.44	1.393	1.44	1.391	1.44
C17–C18	1.394	1.42	1.394	1.43	1.395	1.42
C18–C19	1.393	1.43	1.394	1.43	1.393	1.43
C14–C19	1.403	1.39	1.400	1.40	1.402	1.39
C15–C20	1.525	0.95	1.511	0.96	1.526	0.95
C20–O4	1.253	1.44	1.249	1.45	1.254	1.43
C20–O5	1.253	1.44	1.266	1.36	1.253	1.44
C6–O1	1.257	1.45	1.256	1.45	1.285	1.28
C11–O2	1.257	1.45	1.256	1.45	1.254	1.47
C4–O3	1.359	0.98	1.359	0.98	1.357	0.98
C9–O3	1.359	0.97	1.359	0.98	1.358	0.38
O4–G(H–O _{int})			1.767	0.07	1.663	0.10
O5–G(H–O _{ter})			1.861	0.05		
O1–G(H–O _{int})			1.685	0.10	1.703	0.08
G(H–O _{int})			0.982	0.67	0.993	0.63
G(H–O _{ter}) _{coord}			0.977	0.69		
G(H–O _{ter}) _{free(1)}	0.965 ^a	0.74 ^a			0.969	0.73
G(H–O _{ter}) _{free(2)}	0.970 ^a	0.73 ^a	0.990	0.64	0.986	0.65

^aFree glycerol

near the crossing observed in Figs. 2, 4, 5, 8 and 9, which may be explained by a change in which complex dominates any given property.

3.5.6 TDDFT

The absorption (i.e., vertical excitations) and emission (i.e., fluorescence from first excited state) spectra were calculated

Table 5 Concentration-corrected glycerol–water solution complexation energies ($\Delta G_{298,\text{sol}}$, kcal/mol, SMD-revDSD-PBEP86-D4/def2-TZVPP//SMD(H_2O)-PBE0/def2-SVP) of the different 1:1 fluorescein–glycerol complexes

	12	23	34	42	49	63	70	100
$a_i b_i$	− 1.3	− 1.7	− 2.0	− 2.1	− 2.2	− 2.4	− 2.5	− 2.8
$a_i b_i$	− 0.8	− 1.2	− 1.5	− 1.6	− 1.7	− 1.9	− 2.0	− 2.3
$a_i b_i b_i$	0.3	− 0.1	− 0.4	− 0.5	− 0.6	− 0.8	− 0.9	− 1.2
$c_i c_i$	0.7	0.3	0.1	− 0.1	− 0.2	− 0.4	− 0.5	− 0.7
$a_i a_i$	0.9	0.4	0.2	0.0	− 0.1	− 0.3	− 0.3	− 0.6
$b_i b_i$	1.9	1.5	1.2	1.1	1.0	0.8	0.7	0.4
$a_i c_i$	1.9	1.5	1.3	1.1	1.0	0.8	0.8	0.5
c_i	2.0	1.5	1.3	1.2	1.0	0.9	0.8	0.5
$a_i b_i b_i(\text{alt})^a$	2.0	1.6	1.3	1.2	1.0	0.9	0.8	0.5
$c_i c_i$	2.1	1.7	1.4	1.3	1.2	1.0	0.9	0.6
c_i	2.2	1.9	1.6	1.5	1.4	1.2	1.2	0.9
$b_i b_i$	2.6	2.2	1.9	1.8	1.7	1.5	1.4	1.1
b_i	2.8	2.4	2.2	2.0	1.9	1.8	1.7	1.5
$a_i a_i(\text{alt})^a$	3.2	2.8	2.6	2.4	2.3	2.1	2.1	1.8
a_i	3.3	2.9	2.6	2.5	2.4	2.2	2.1	1.8
$a_i a_i$	3.6	3.2	2.9	2.8	2.7	2.5	2.4	2.2
$a_i d_i$	3.9	3.5	3.3	3.1	3.0	2.8	2.8	2.5
d_i	5.5	5.1	4.9	4.7	4.6	4.5	4.4	4.2
d_i	7.3	6.9	6.6	6.5	6.4	6.3	6.2	6.0

^aIn these two cases, a second (alternate) geometry was obtained, with the higher-energy conformer given the “alt” tag

Table 6 Boltzmann probabilities (%) of the different 1:1 fluorescein–glycerol complexes based on the complexation energies in Table 5

	12	23	34	42	49	63	70	100
$a_i b_i$	58.8	60.9	61.7	62.0	62.2	62.5	62.7	63.0
$a_i b_i$	25.3	26.2	26.5	26.6	26.7	26.8	26.8	26.9
$a_i b_i b_i$	4.1	4.3	4.3	4.3	4.3	4.4	4.4	4.3
$c_i c_i$	1.9	2.0	2.0	2.0	2.0	2.0	2.0	2.0
$a_i a_i$	1.6	1.6	1.7	1.7	1.7	1.7	1.7	1.7
$b_i b_i$	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
$a_i c_i$	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2
c_i	0.2	0.3	0.3	0.3	0.2	0.2	0.2	0.2
$a_i b_i b_i(\text{alt})^a$	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
$c_i c_i$	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
c_i	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1
$b_i b_i$	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
b_i	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0
$a_i a_i(\text{alt})^a$	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
a_i	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$a_i a_i$	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$a_i d_i$	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
d_i	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
d_i	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Free FS^{2-}	6.7	3.4	2.2	1.8	1.5	1.1	0.9	0.6

The energy of $\text{FS}^{2-} \cdot 4\text{H}_2\text{O}$ is taken as 0.0 kcal/mol for the purpose of the Boltzmann distribution

^aIn these two cases, a second (alternate) geometry was obtained, with the higher-energy conformer given the “alt” tag

Table 7 Iterative Hirshfeld partial charges (SMD-PBE0/def2-SVP, with the charges on the hydrogen atoms summed into the charges on the heavy atoms) on various parts of FS²⁻ and selected complexes

Fragment	FS ²⁻	$a_t b_t$	$a_t b_t b_t$	$c_t c_i$	$a_t b_t b_t + c_t c_i$	$a_t b_t b_t + c_t c_i + c_t c_i$
Xanthene ^a	− 1.06	− 1.05	− 1.04	− 0.90	− 0.87	− 0.72
CO ₂ [−]	− 0.83	− 0.68	− 0.65	− 0.82	− 0.65	− 0.65
Ph	− 0.11	− 0.08	− 0.08	− 0.11	− 0.07	− 0.06
Glycerol		− 0.20	− 0.24	− 0.17	(1) − 0.23 (2) − 0.17	(1) − 0.24 (2) − 0.16 (3) − 0.17

^aIncluding the two exocyclic oxygen atoms

Fig. 10 Plot of the distribution of each type of complex versus glycerol concentration with the associated logarithmic regressions

using TDDFT. The τ HCTHhyb hybrid functional was chosen based on its performance for valence π – π^* transitions in various benchmark tests [69–74]. It should be noted from the start that the prediction of absorption and emission spectra of large, organic molecules, and particularly in solution, is a significant challenge. The τ HCTHhyb functional was chosen based on its performance in various benchmark studies, which primarily consider absorption, where even for the best performing methods there is a deviation from experiment. What is generally considered is the observed trends and whether they agree and explain the experimental observations and not a rigorous quantitative agreement with the experimental spectra.

As noted above, in order to obtain more reliable spectra for species that form hydrogen bonds, FS²⁻•4H₂O is used instead of unsolvated fluorescein. Explicit water molecules were not added to the fluorescein–glycerol complexes since

the glycerol itself forms the hydrogen bonds. The final spectra shown in Fig. 11 are Boltzmann-weighted sums of the spectra of each complex, where each spectrum is approximated as a Gaussian centred on the frequency of each absorption (ν_i):

$$I(\nu) = \sum_i p_i \cdot f_i \cdot \exp\left(-\frac{(\nu - \nu_i)^2}{2\sigma^2}\right)$$

where $I(\nu)$ is the intensity (arbitrary units) at wavelength ν , p_i is the Boltzmann weight, f_i is the oscillator strength (from the TDDFT calculation) and σ is the standard deviation, which is proportional to the full-width at half-maximum height (FWHM) according to $\text{FWHM} = 2\sqrt{2 \ln(2)}\sigma$; [102] a FWHM of 5 nm was chosen. The oscillator strength f_i is a measure of the maximum intensity: $\epsilon_i^{\max} = \frac{f_i \cdot \sqrt{\pi} \cdot N_A \cdot e^2}{1000 \ln(10) \cdot m_e \cdot c^2 \cdot \sigma}$,

Fig. 11 Calculated absorption (solid lines) and emission (dashed lines) spectra for different concentrations of glycerol, using a full-width at half-maximum (FWHM) of 5 nm

where N_A is Avogadro's number, e is the charge on an electron, m_e is the mass of an electron and c is the speed of light [103]. As noted above, ν_i and f_i are taken as weighted averages of the values in water and neat glycerol. Because the difference between f_i and $\epsilon_i^{\max}$ is just a scale factor that is independent of the specific absorption or emission, which in effect would be to scale $I(\nu)$, for simplicity only f_i is used. The data for each complex is provided in Table S8.

The absorption spectra qualitatively resemble the experimental spectra in Fig. 1 (the maxima are summarized in Table S9). There is a shift in frequency and differences in the intensities of the bands, but overall key features are preserved. As the concentration of glycerol increases, there is a decrease in intensity of the absorption at ~ 500 nm and formation of a second band at ~ 490 nm. Based on the absorptions of the different complexes (Table S8), the first band can be associated with 1:1 complexes, which are predominant in low glycerol concentrations, while the second band that forms is associated with 1:2 complexes, which forms as the concentration of glycerol increases.

From the data in Table S8, the absorption wavelengths ν follow the trend $\text{FS}^{2-} (\text{FS}^{2-} \cdot 4\text{H}_2\text{O}) > 1:1 > 1:2 > 1:3$. The absorption is associated with the first excited state, and the natural transition orbitals (NTOs) for this excitation

(Fig. 12), which show the source and destination of electron density during the electronic excitation, indicate that this is a π – π^* excitation on the xanthene fragment. (This is also important since our choice of functional was based on benchmark evaluations of valence (π – π^*) excitations.) This explains the observed trend in ν : the formation of hydrogen bonds shifts electron density to the glycerol ligands. This shift in electron density is also apparent from the iterative Hirshfeld charges (Table 7): coordination of glycerol reduces the negative charge on the xanthene system, which increases with each added glycerol, and adds it to the glycerol molecules. This shift in electron density will help stabilize the HOMO making the HOMO–LUMO gap larger, and this effect will be larger when the glycerol coordinates to the xanthene ring, as occurs in the 1:2 and 1:3 complexes.

The experimental and calculated emission spectra likewise are qualitatively similar. Both have a decrease in intensity with increasing glycerol concentration, but the later has structure that is not present in the former. However, this could be an artefact of the value of FWHM used, and using a larger values (Figure S7) gives broadening more resembling the experimental spectrum. Nonetheless, the added structure provides insight into the cause of the broadening—specifically initial formation of 1:1 complexes (~ 535 nm), followed

Fig. 12 τ HCTHhyb/def2-TZVPP natural transition orbitals (NTOs) for (top-to-bottom) FS^{2-} , a_1b_1 , $a_1b_1 + c_1c_1$ and $a_1b_1 + c_1c_1 + c_1c_1$. Green and red colours correspond to the positive and negative phases of the orbitals

by 1:2 complexes (~ 540 nm) at higher glycerol concentrations, and finally 1:3 complexes (~ 525 nm) at the highest glycerol concentrations.

4 Conclusions

The spectro-photo-fluoroscropy features of fluorescein, which was chosen as a representative fluorophore, were found to be strongly dependent on the glycerol concentration in aqueous glycerol. Glycerol was found to impact both the ground and first excited states of fluorescein, to quench and modify both absorption and emission spectra, to affect the fundamental electrical dipoles of either the ground or first excited singlet states, and to lower FLT and quantum yield. In addition, the Strickler–Berg relation was proposed as an indicator for deformations in a fluorophore solute caused by its hosting media.

Employing computational chemistry, the predicted absorption and emission (fluorescence) spectra were found

to correlate with the experimental data. The experimentally observed change in spectral properties at a glycerol concentration of $\sim 40\%$ was found to result from a shift in the dominant species in solution from a 1:1 fluorescein:glycerol complex to a 1:2 complex at this approximate concentration.

Hence, considering glycerol as an inert medium with which one can adjust viscosity, refraction index and polarizability is a naïve assumption in the view of the authors. Last but not least, it is the aim of this group to extend the present study to include other fluorophores and solvents, and initial results indicate that glycerol is involved in diverse interactions and reactions (data not shown).

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s43630-021-00096-w>.

Acknowledgements This study was endowed by the Bequest of Moshe-Shimon and Judith Weisbrodt.

Author contributions HF: M.Sc. student who performed the study and helped write the manuscript. MAI: carried out the computational chemistry researched and helped write the manuscript. DF: supervised the lifetime measurements. SM: biochemist who was in charge of solution preparation. NZ: microbiologist who was in charge of the biotechnological aspects of the study and help write the manuscript. EA: helped in macroscopic measurements of the fluorescent solutions. MD: supervised the entire study and helped write the manuscript.

Funding This study was endowed by the Bequest of Moshe-Shimon and Judith Weisbrodt.

Declarations

Conflicts of interest There are no conflicting or competing interests to declare.

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