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Spin the light off: rapid internal conversion into a dark doublet state quenches the fluorescence of an RNA spin label†

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The spin label ζ_m and the fluorophore ζ_m^f are close isosteric relatives: the secondary amine ζ_m^f can be easily oxidized to a nitroxide group to form ζ_m . Thus, both compounds can serve as EPR and fluorescence labels, respectively, and their high structural similarity allows direct comparison of EPR and fluorescence data, e.g. in the context of investigations of RNA conformation and dynamics. Detailed UV/vis-spectroscopic studies demonstrate that the fluorescence lifetime and the quantum yield of ζ_m^f are directly affected by intermolecular interactions, which makes it a sensitive probe of its microenvironment. On the other hand, ζ_m undergoes effective fluorescence quenching in the ps-time domain. The established quenching mechanisms that are usually operational for fluorophore-nitroxide compounds, do not explain the spectroscopic data for ζ_m . Quantum chemical calculations revealed that the lowest excited doublet state D_1 , which has no equivalent in ζ_m^f , is a key state of the ultrafast quenching mechanism. This dark state is localized on the nitroxide group and is populated via rapid internal conversion.

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Introduction

The understanding of the mechanisms underlying action of biologically relevant macromolecules such as RNA is closely related to its structure and dynamics. Spectroscopic experiments play a pivotal role in gaining insights into these structural, dynamical and mechanistic aspects. The advancement of such studies is driven by both better instrumentation and improved labels that are used as spectroscopic reporter groups.^{1–13} An example of a very sophisticated label is ζ .^{14–20} It is a rigid nitroxide that is a deoxy-cytidine analogue and can thus form a stable base-pair with guanine after incorporation into DNA. It has been used to study DNA by electron paramagnetic resonance (EPR) spectroscopy. The rigidity of the label suppresses the configurational ambiguity of most other, usually flexible EPR spin-labels. This allows both to measure distances within

DNA^{16,18} and relative orientations between different helical elements.²⁰

Another very useful aspect of ζ is that the reduction of the nitroxide group turns it fluorescent.¹⁴ ζ is a so-called fluorophore-nitroxide (FNRO•) compound, where a nitroxide is directly linked to a fluorophore. The nitroxide acts as efficient fluorescence quencher within the FNRO• compound.^{21–23} In effect, ζ is bifunctional: it can be utilized for EPR and fluorescence spectroscopy, two distinct and complementary experimental techniques suitable for biophysical research.^{24,25} In fact, the relation between the two compounds is so close that the fluorescent form, called ζ^f , is a precursor in the synthesis of ζ . The two compounds are for all practical purposes isosteric to each other. The amine ζ^f has been used as a fluorescent label for steady-state fluorescence experiments.^{24–28} ζ^f is very sensitive to its microenvironment, as demonstrated by its ability to detect mismatches within duplex DNA and to identify the mismatched counter base.^{25–28}

We are interested in applying fluorescence spectroscopy to investigate the structure and dynamics of functional RNAs. ζ_m is a derivative of the spin label ζ that contains a 2'-methoxy group (Fig. 1), and it has been prepared and incorporated into RNA.^{29,30} In accordance with expectations, the precursor in its synthesis, ζ_m^f (Fig. 1), is fluorescent as well. Therefore, it is a promising probe for studying RNA. To conduct and understand

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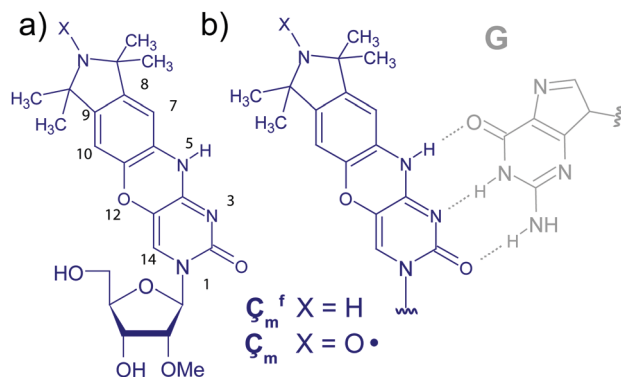


Fig. 1 Structure (a) and base pairing properties (b) of C_m and C_m^f with guanine (G).

time-resolved absorption and fluorescence studies of conformational RNA dynamics with the help of C_m^f , it is necessary to know more about the photochemical properties of the fluorescent nucleoside itself. In addition, characterization of C_m allows comparison between the quenched (paramagnetic) and the unquenched (diamagnetic) fluorophore yielding insights into the intramolecular fluorescence quenching (IFQ) mechanism of this specific FNRO• compound.

The excited states and the possible fluorescence quenching mechanisms (inter- and intramolecular ones) for a variety of FNRO• compounds have been extensively studied and discussed during the last 40 years.^{22,31–51} Since the first studies,^{31–34} some different mechanisms have been described in this context: excitation-energy transfer (EET) of Förster (FT) and Dexter (DT) type, electron transfer (ET), electron-exchange induced enhanced intersystem crossing (EISC) and enhanced internal conversion (EIC) to the electronic ground state. All of these mechanisms may, in general, contribute to the quenching rate k_q of a compound (cf. eqn (1)).

$$k_q = k_{ET} + k_{FT} + k_{DT} + k_{EISC} + k_{EIC} \quad (1)$$

To understand the photochemistry of C_m and C_m^f in general and in particular the fluorescence quenching of C_m , we present here a detailed steady-state and time-resolved UV/vis-spectroscopic and theoretical study of both compounds. During the course of this study, we found none of the above mentioned mechanisms to be correct for C_m . Instead, we found a rapid internal conversion (IC) into a dark, excited doublet state, which has no equivalent in C_m^f , as quenching mechanism for this FNRO• compound. To the best of our knowledge, such a quenching mechanism was not described for FNRO• compounds up until now.

Experimental

Materials and methods

Sample preparation. The synthesis of both molecules was carried out according to a published procedure.²⁹ C_m was additionally purified *via* high-performance liquid chromatography (HPLC). C_m and C_m^f are both soluble in water, although

the solubility of C_m^f is superior to C_m . Unless stated otherwise, the samples were dissolved in a 20 mM sodium cacodylate buffer (Nacacodylate) at pH 7.4.

For studies of solvent-dependence, the samples were dissolved in EtOH (p.a.). Aliquots were removed, the EtOH evaporated and the residues were dissolved in the solvents of choice (dimethyl sulfoxide/DMSO, acetonitrile, toluene, deuterium oxide; each p.a.).

Steady-state spectroscopy. Steady-state absorption spectra were recorded in 10 mm × 4 mm UV-grade quartz cuvettes (29-F/Q/10, Starna GmbH, Pfungstadt, Germany) on a JASCO V-650 spectrometer (JASCO Germany GmbH, Groß-Umstadt, Germany). The spectra were offset corrected and normalized. Emission spectra were recorded in 10 mm × 4 mm UV-grade quartz cuvettes (29-F/Q/10, Starna GmbH) with a JASCO FP 8500 fluorescence spectrometer. Prior to normalization, the spectra were corrected for offset, absorption and reabsorption artifacts as well as the spectral characteristics of the experimental equipment. The JASCO FP 8500 spectrometer was equipped with a 100 mm integrating sphere (ILF-835, JASCO) and used for absolute quantum yield determinations of the samples.

Femtosecond transient absorption spectroscopy. (TAS) was performed with a home-built pump-probe setup.¹⁰ As source for the femtosecond laser pulses, an oscillator-amplifier system (CPA-2001, Clark-MXR, Michigan, USA) operating at a repetition rate of 1 kHz (775 nm, pulse width of 150 fs) was used. Excitation at 388 nm was obtained by second harmonic generation (SHG) of the 775 nm beam in a beta-barium borate (β -BaB₂O₄, BBO) crystal. Probe pulses with a spectral range from 380 nm to 680 nm were generated in a CaF₂ crystal. These pulses were split into a signal and a reference beam. For detection, each probe pulse was guided to a spectrograph (HR320, HORIBA, Kyoto, Japan). The signals were detected with the help of a photodiode array combined with a signal processing chip (S8865-128, Hamamatsu Photonics, Hamamatsu, Japan) and a driver circuit (C9118, Hamamatsu Photonics). For digitalization a data acquisition card (NI-PCI-6120, National Instruments, Austin, USA) was used. The samples were prepared in UV-grade quartz cuvettes with 1 mm optical path length (21/Q/1, Starna GmbH). In order to prevent possible reexcitation of already excited molecules, the cuvette was moved in the two directions perpendicular to the excitation pulses. The sample was excited with pulse energies of ≤ 120 nJ and relative pump/probe polarization in the magic angle (54.7°) to eliminate anisotropy.⁵² Data processing and global lifetime analysis (GLA) was performed with the OPTIMUS 2.08 software.⁵³

Time-resolved fluorescence measurements. The fluorescence lifetimes were measured with a partly home-built time-correlated single photon counting (TCSPC) setup as described previously.¹¹ For excitation, a mode-locked titanium-doped sapphire (Ti:Sa) laser (Tsunami 3941-X3BB, Spectra-Physics, Darmstadt, Germany) was pumped by a 10 W continuous wave diode pumped solid state laser (Millennia eV, Spectra-Physics, 532 nm). The Ti:Sa Laser allowed the tuning of the excitation wavelength to 775 nm at a repetition rate of 80 MHz. With the help of an acousto-optic modulator, the repetition rate was reduced to 8 MHz and the excitation wavelength of 388 nm was obtained by SHG in a BBO

crystal (frequency doubler and pulse selector, Model 3980, Spectra-Physics). Excitation pulses of about 0.1 nJ at 388 nm were applied to the sample. The sample was prepared in a 10 mm × 4 mm quartz cuvette (29-F/Q/10, Starna GmbH) with a fixed temperature of 20 °C. Emission filters (GG395, GG400, Schott AG, Mainz, Germany) suppressed excitation stray light. The instrument response function (IRF, FWHM 200 ps) was obtained without emission filters using a TiO₂ suspension as scattering sample. For spectral assignments, three different notch filters (436 nm, 464 nm, 575 nm) were used (Fig. S1a, ESI†). For single-photon detection, a photomultiplier tube (PMT, PMA-C 182-M, PicoQuant, Berlin, Germany) and a TimeHarp 260 PICO Single PCIe card (PicoQuant) was used. Multi-exponential fitting was carried out with FluoFit Pro 4.6 (PicoQuant).⁵⁴

Phosphorescence lifetime measurements. Phosphorescence lifetime measurements were made with a home-built phosphorescence setup. For excitation, a mounted CW 365 nm LED (M365L2, Thorlabs, Newton, USA) was used. The excitation light was chopped by a modified chopper wheel (MC200-EC, Thorlabs) with a 0.5 Hz repetition rate and was led into a cryostat (DN1704, Oxford Instruments, Abingdon, UK). The sample was prepared in a shortened NMR quartz tube (qtz300-5-7, Deutero GmbH, Kastellaun, Germany) which was fixed on the sample rod of the cryostat. The emission signal was collected in 90° angle to the excitation and was sent again through the chopper wheel. Thus, the excitation stray light and the direct fluorescence was blocked. Additionally, emission filters (GG400, GG420, Schott AG) were blocking stray light. The chopped emission signal was passed through a Czerny–Turner monochromator and focused onto a PMT (H10721-01, Hamamatsu Photonics). The PMT signal was measured with a 1 GHz digital storage oscilloscope (TDS 5104, Tektronix, Beaverton, USA). The data acquisition was triggered by the excitation stray light with the help of a high-speed photodetector (DET10A/M, Thorlabs). At least 100 excitation cycles were averaged to obtain a single phosphorescence transient. Data processing and the exponential fitting was carried out with OriginPro 2016G (OriginLab Corporation, Northampton, USA).

Quantum-chemical calculations. Quantum-chemical calculations of $\zeta_{\text{m}}^{\text{f}}$ and ζ_{m} were performed using the program package Q-Chem 4.4.⁵⁵ The vertical excitation energies as well as the ground and excited-state geometries were computed using density-functional theory⁵⁶ (DFT) and its time-dependent variant⁵⁷ (TD-DFT). The calculations employed the ω -B97x⁵⁸ functional including a dispersion correction in 3rd generation (-D3)⁵⁹ and the 6-31g* basis set.^{60–68}

Furthermore, ζ_{m} was investigated using the spin-flip (SF) TD-DFT method which is known to overcome problems of spin-contamination of the ground state wave functions in single-reference methods.^{69–74} In general, a stable triplet state was used as reference for the parent method within the SF ansatz. From this reference, an excitation subspace was built in which one of the spins is flipped (in most cases, the excitation exhibits a change in spin of $\Delta m_s = -1$ from a high-spin $\alpha\alpha$ triplet). This SF excitation subspace contains all possible (singlet and triplet) $m_s = 0$ determinants.

Results and discussion

Steady-state spectroscopy

The steady-state absorption and emission spectra of $\zeta_{\text{m}}^{\text{f}}$ and ζ_{m} in aqueous solution (Fig. 2) do not differ significantly from the corresponding ζ^{f} spectra.¹⁴ The absorption maximum of ζ_{m} and $\zeta_{\text{m}}^{\text{f}}$ can be found at 27 630 cm⁻¹ (362 nm).

The fluorescence maximum of both compounds can be found at 21 740 cm⁻¹ (460 nm). Accordingly, absorption and emission maxima are shifted by roughly 5890 cm⁻¹ (100 nm) and only a small overlap between the absorption and the emission spectra exists. It is particularly interesting to compare the fluorescence-quantum yields of $\zeta_{\text{m}}^{\text{f}}$ and ζ_{m} : $\zeta_{\text{m}}^{\text{f}}$ has a quantum yield of $\phi = 38\%$, whereas ζ_{m} has a quantum yield of only $\phi < 1\%$, which demonstrates the strong quenching capability of the nitroxide group. At 77 K, it is possible to measure phosphorescence spectra, too. The phosphorescence spectra of both compounds show great similarity to each other. The emission maximum of ζ_{m} can be found at 19 600 cm⁻¹ (510 nm), while the phosphorescence spectrum of $\zeta_{\text{m}}^{\text{f}}$ is slightly broadened and the emission maximum is red shifted to 18 850 cm⁻¹ (530 nm). Accordingly, there is a shift between the fluorescence and the phosphorescence maxima for ζ_{m} and $\zeta_{\text{m}}^{\text{f}}$ of about 2140 cm⁻¹ (50 nm) and 2890 cm⁻¹ (70 nm), respectively.

Time-resolved fluorescence measurements

The fluorescence lifetimes of both compounds were determined by TCSPC at room temperature (Fig. 3 and Table S1, ESI†). For $\zeta_{\text{m}}^{\text{f}}$, the fluorescence decay can be described with a triexponential decay function, where a single time constant of 300 ps, with a negative amplitude (τ_{pop}), describes the population of the emitting states. The time constants $\tau_1 = 4.1$ ns and $\tau_2 = 1.4$ ns describe the excited state decay. All time constants result in an intensity-weighted average lifetime⁷⁵ (eqn (2)) of $\tau_{\text{av}} = 4.1$ ns.

$$\tau_{\text{av}} = \frac{\sum_i A_i \tau_i^2}{\sum_i A_i \tau_i} \quad (2)$$

For ζ_{m} , a very fast fluorescence decay component can be noticed as a peak at the beginning of the decay. Such a peak results from a signal decay that is faster than the width of the IRF (FWHM 200 ps). A fit of the decay, which takes into account the IRF, yields

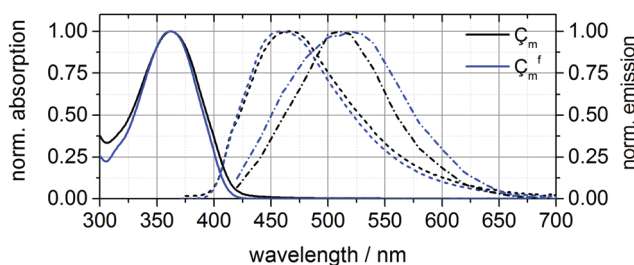


Fig. 2 Normalized steady-state absorption (solid lines), fluorescence (dashed lines) and low temperature phosphorescence spectra (dashed and dotted lines) of ζ_{m} (black) and $\zeta_{\text{m}}^{\text{f}}$ (blue).

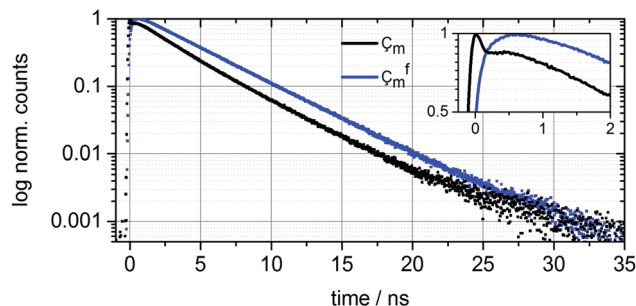


Fig. 3 Time-resolved fluorescence measurements of C_m (black) and C_m^f (blue). A close-up of the first 2 ns is shown as inset.

a lifetime of $\tau_3 = 3$ ps. This represents, however, only an upper limit for the true decay time. The fast signal decay is followed by a buildup (230 ps) of a much weaker emission signal. This signal then decays with two lifetimes of $\tau_1 = 4.3$ ns and $\tau_2 = 2.3$ ns. Thus, the overall C_m decay can be described with $\tau_{av} = 200$ ps (see eqn (2) and Table S1, ESI†) and consists of a strong ps-component and two relative weak ns-components. The ns-components are similar to the decay constants of C_m^f . However, sample contamination by C_m^f can be excluded, because C_m was purified with the help of HPLC (mobile phase: 0.1 M TEAA-buffer + acetonitrile) (gradient 0–40% in 25 min). It was possible to separate C_m and C_m^f clearly from each other, because of different retention times (C_m : 22.5 min; C_m^f : 16.5 min).

The contribution of the fast (τ_3) and the slow (τ_1, τ_2) decay components to the overall fluorescence decay of C_m differ spectrally (Fig. 4). Therefore, it is possible to separate the components. The fast decay component (τ_3) dominates in the high-energy shoulder and the slower ones (τ_1, τ_2) in the low-energy parts of the fluorescence spectrum. Without the overlaying fast component, the buildup of the slower decay components described above is more clearly visible.

Solvent-dependent fluorescence quantum yield and lifetime measurements

Gardarsson *et al.* previously showed a strong dependence of the C_m^f fluorescence on the pH value of the solvent.²⁸ To complement these observations concerning possible solvent interactions the quantum yields and fluorescence lifetimes of C_m and C_m^f were

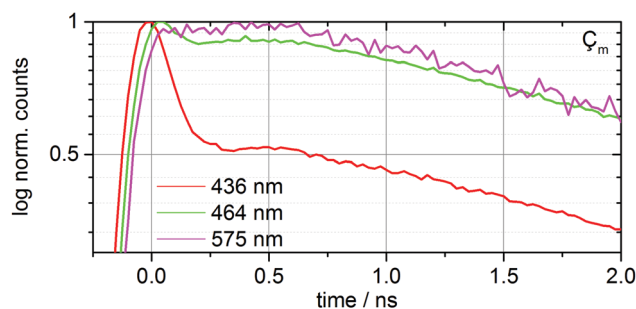


Fig. 4 Time-resolved fluorescence measurements of C_m at different detection wavelengths. The indicated values refer to the center wavelengths of the respective notch filters (for exact spectra see Fig. S1, ESI†).

determined in a series of different aprotic solvents (Table S1 and Fig. S2, ESI†). These solvent interactions were analyzed relative to the polarity index introduced by Snyder.⁷⁶

Both quantities vary strongly with the properties of the solvent molecules. Nevertheless, it was not possible to identify a clear trend within this dataset. Only for C_m^f , the fractional intensity of τ_1 increases while the fractional intensity of τ_2 decreases with increasing polarity index, respectively, with increasing intermolecular interactions.

To elucidate the influence of a possible proton transfer on the fluorescence-quenching mechanism, we also measured the fluorescence lifetime and the fluorescence quantum yield of C_m and C_m^f in deuterium oxide (D_2O): Comparing C_m^f in H_2O and D_2O shows a characteristic isotope effect. τ_{av} is increased in D_2O by a factor of 1.3 compared to H_2O . The same factor can be found by the comparison of the fluorescence-quantum yield. For C_m , it can be noticed that the different decay components are differently affected by the solvent exchange. While there is no noticeable effect on the ps-component (τ_3) of the C_m fluorescence decay, there is again an isotope effect for the signal buildup (factor 1.5) and for the longest decay component τ_1 (factor 1.2). Therefore, it seems that the main quenching process of C_m is not effected by the H_2O to D_2O solvent exchange. But the buildup and decay of the long-lived components is slowed down in D_2O .

Femtosecond transient absorption spectroscopy

For C_m^f , three different transient absorption signals between 370 nm and 650 nm (Fig. 5a) can be identified: there is a positive band between 370 nm and 490 nm and another one between 590 nm and 680 nm. The two bands are attributed to excited-state absorption (ESA). Between the ESA signals, from 490 nm to 590 nm, a negative signal was detected. All three signals appear instantaneously after photoexcitation. As the negative signal coincides spectrally with the steady-state fluorescence despite the fact that it is partially compensated by the neighboring ESA, it is identified as stimulated emission (SE). Due to the high-energy ESA, it is also not possible to see the ground state bleach (GSB) as a negative signal in the transient card. All signals gain intensity within the first 10 ps of the measurement. Thereafter, there is a decay of the signals which is not completed within the measurement window (Fig. S6a and S7a, ESI†).

To determine the dynamics of the fluorophore C_m^f after photoexcitation a multiexponential decay function was used to fit the data (Fig. 5a) by global analysis. It is possible to describe the data with three time constants. The corresponding DAS are shown in Fig. 5c. The smallest time constant of 1.4 ps describes a signal buildup that is most likely due to an internal relaxation process of the molecule after excitation. The slower signal decay can be described with two time constants of 170 ps and 4.1 ns. The slowest time-constant matched the fluorescence lifetime that was determined by TCSPC experiments. Due to the limited time window of the experiment, it is not possible to observe the full signal decay, which is why the value of 4.1 ns was determined with an uncertainty of about $\pm 10\%$.

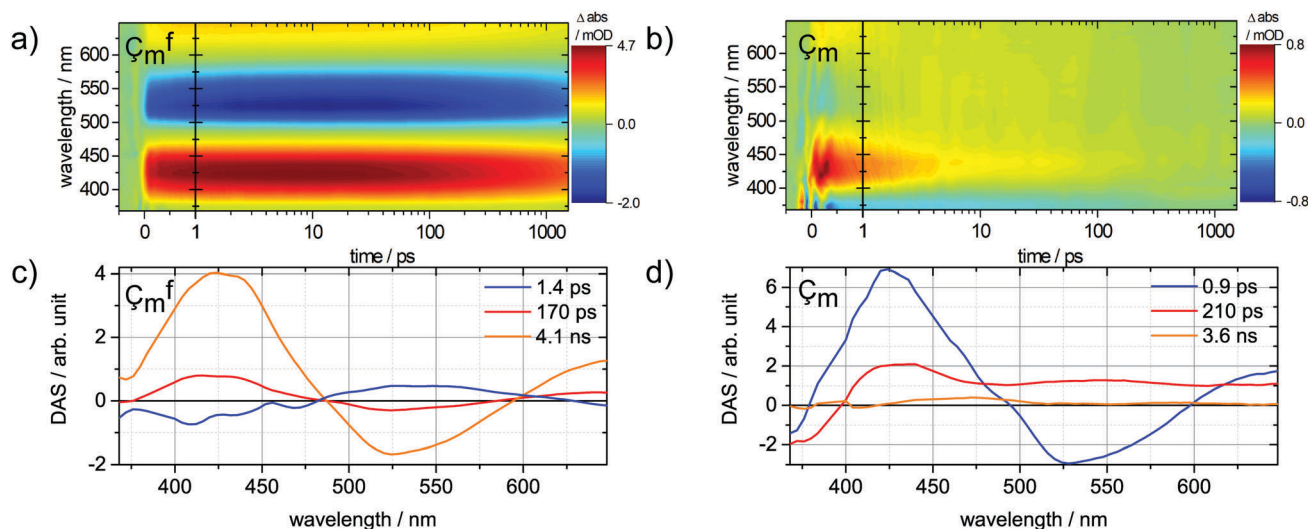


Fig. 5 Transient absorption measurement of C_m^f and C_m . Transient cards of C_m^f (a) and C_m (b). Decay associated spectra (DAS) of C_m^f (c) and C_m (d).

The spin label C_m shows four transient signal components between 370 nm and 650 nm (Fig. 5b). Altogether the spectral positions of the bands do not differ significantly from the positions in C_m^f . Accordingly there is a broad ESA from 400 nm to 680 nm which is overlayed by an even faster decaying SE between 490 nm and 590 nm. The amplitude of the ESA and SE in C_m is much weaker than in C_m^f . Because of that, there is also a negative signal of the GSB between 370 nm and 400 nm, which cannot be seen in the case of C_m^f .

The most striking difference between C_m and C_m^f is that the signal decay of C_m is much faster. The transient data of C_m can be fitted by a sum of three exponential functions with the time constants 0.9 ps, 210 ps and 3.6 ns. Here the 0.9 ps time constant describes the complete signal decay of the SE and the main part of the ESA. This can be seen also in the transients (Fig. S7b, ESI†): the SE signal is strong immediately after the photoexcitation but decays fast within the first picosecond. Altogether, the SE decay of the nonfluorescent C_m is about three orders of magnitude faster than the signal decay of C_m^f . Therefore, fluorescence is quenched within the time range of 0.9 ps.

After fluorescence quenching, relatively weak ESA and GSB persist. The decay of these signals can be described by two further time constants, 210 ps and 3.6 ns. As can be seen in the DAS (Fig. 5d), the amplitude of the largest time constant is very small. Nevertheless, there is a long-lived positive component extending to the end of the measurement window (see time slices in Fig. S6b, ESI†). Here, a slight bathochromic shift of the largest ESA signal at around 420 nm is also noticeable.

Taken together, and in accordance with quantum yield and time-resolved fluorescence data, the fast SE decay results in an efficiently quenched fluorescence.

Quantum-chemical calculations

In Table 1, the vertical excitation energies of C_m^f and C_m are compiled which have been calculated with respect to their

Table 1 Vertical excitation energies (ω), spin state characterized by $\langle \hat{S}^2 \rangle$ and corresponding oscillator strength (f) of the lowest excited states of C_m^f and C_m calculated at the S_0 and D_0 ground state geometries, respectively

State	E/eV [nm]		$\langle \hat{S}^2 \rangle$	f	
	C_m^{fb}	C_m^a		C_m^{fb}	C_m^a
D_1	—	4.02 [308]	1.70	—	0.00
S_1/D_2	4.36 [284]	4.32 [287]	1.09	0.30	0.29
S_2/D_3	4.97 [249]	6.32 [196]	0.91	0.06	0.07
T_1/Q_1	3.09 [401]	2.91 [426]	3.75	0.00	0.00
T_2/Q_2	3.73 [323]	3.58 [346]	2.75	0.00	0.00

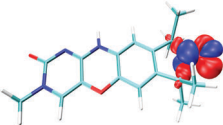
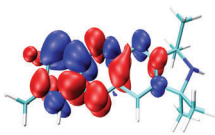
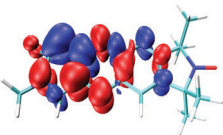
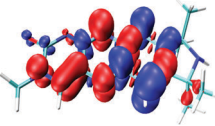
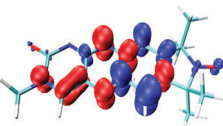
^a Calculated with SF-TD-DFT method and a quartet reference. ^b Calculated using conventional TDDFT. ^c Expected values for spin-pure doublet and quartet wave functions are 0.75 and 3.75, respectively ($S^2 = S(S+1)$).

corresponding singlet (S_0) and doublet (D_0) ground states, respectively. For the calculation of C_m , the quartet Q_1 -state was used as reference within SF-TDDFT. In general, the SF single-excitation subspace contains all doublet determinants, which allows for the construction of all doublet- (also D_0) and quartet states within the single-excitation space of TDDFT leading to a balanced and consistent description of radicaloid systems such as C_m .^{69,71,72} For consistency, the ω -B97x-D3 functional was used combined with the 6-31g* basis set.

According to our calculations, the first excited state (S_1) of C_m^f exhibits an excitation energy 4.36 eV and an oscillator strength of 0.3. Hence, the S_1 -state corresponds to the peak at 362 nm in the experimental absorption spectrum (Fig. 2). The spin label C_m , exhibits an identical, computed state (D_2) at 4.32 eV with an oscillator strength of 0.29, which is also visible at exactly the same position in the experiment as the one of C_m^f (S_1 -state; Fig. 2), at 362 nm. Analysis of the difference densities characterizing these two excited states reveals their electronic structure is identical and typical for π - π^* states (Table 2).

However, C_m possesses an additional state D_1 , with a computed vertical excitation energy of 4.02 eV and no oscillator strength, which has no analogue in C_m^f . It should thus not be

Table 2 Difference densities of the lowest excited states of $\zeta_{\text{m}}^{\text{f}}$ and ζ_{m} . The figures demonstrate the change in electron density upon excitation from the electronic ground state to the corresponding excited state. The character of the transition is given in round brackets

State	$\zeta_{\text{m}}^{\text{f}}$	ζ_{m}
$D_1(n-\pi^*)$	—	
$S_1(\pi-\pi^*)/D_2(\pi-\pi^*)$		
$S_2(\pi-\pi^*)/D_3(\pi-\pi^*)$		

visible in the experimental absorption spectrum of ζ_{m} . Not surprisingly, D_1 corresponds to a typical $n-\pi^*$ state localized fully at the nitroxide group of ζ_{m} (Table 2).

The next higher excited states of $\zeta_{\text{m}}^{\text{f}}$ and ζ_{m} are again identical from an electronic structure point of view: they correspond to the same $\pi-\pi^*$ transition and exhibit similarly small oscillator strengths. However, the state of ζ_{m} has a higher vertical excitation energy. For this investigation, these states are however not relevant.

To address the fluorescence properties of $\zeta_{\text{m}}^{\text{f}}$ and ζ_{m} , the equilibrium structures of the S_1 - and D_2 -states have been optimized. Since these states are localized at the rigid aromatic site of the labels, the equilibrium structures remain planar and only small geometric changes occur. The computed vertical excitation energies at the excited-state equilibrium geometries, *i.e.* the fluorescence energies of $\zeta_{\text{m}}^{\text{f}}$ and ζ_{m} out of these S_1/D_2 -state minima are 3.66 eV and 3.69 eV relating to a Stokes shift of ≈ 0.7 eV, which agrees very well with the measured one of 0.73 eV (5890 cm^{-1}). Hence, the observed fluorescence signal of $\zeta_{\text{m}}^{\text{f}}$ and ζ_{m} clearly originates from the S_1 and D_2 -states, respectively. The computed static dipole moment of this state is 5.1 Debye, which is almost identical to the one of the ground state with 5.3 Debye, exhibiting no indication of charge transfer.

Quenching mechanism

In the following discussion of the fluorescence quenching mechanism of ζ_{m} we start with a summary of the main results. On the basis of the characterization of the fluorophore $\zeta_{\text{m}}^{\text{f}}$, we were then able to discuss a model for the photodynamics of $\zeta_{\text{m}}^{\text{f}}$ and ζ_{m} . In the case of ζ_{m} the additional D_1 -state is discussed in detail and a quenching pathway is proposed. After that, this quenching pathway is compared with other known quenching mechanisms, to substantiate the proposal.

Summary of results. In the present UV/vis spectroscopic study of the RNA labels $\zeta_{\text{m}}^{\text{f}}$ and ζ_{m} we were able to observe

the following spectroscopic key properties of the compounds: $\zeta_{\text{m}}^{\text{f}}$ is a fluorophore with a fluorescence quantum yield of 38% and a fluorescence lifetime in the ns-range. Remarkably, the fluorescence decay is not monoexponential. In fact, there are two fluorescence decay components in the ns-range. Both components show a notable solvent dependence.

ζ_{m} , on the other hand is virtually nonfluorescent, with a fluorescence quantum yield below 1% and a fluorescence lifetime in the ps-range. The rapid and efficient fluorescence quenching is solvent independent. However, fluorescence lifetime measurements show two very weak decay components, which were very similar to the main decay components of $\zeta_{\text{m}}^{\text{f}}$. Apart from this, transient absorption measurements revealed a weak, long lived ($> 1.5\text{ ns}$) ESA signal.

In contrast to the large differences in the fluorescence quantum yields and the photodynamics, there is no difference in the shape of the steady-state spectra of both compounds. This is basically also true for the vertical excitation energies that were obtained by quantum chemistry calculations. This high structural and spectral similarity, paired with a massive fluorescence quenching is quite common for FNRO• compounds.²² Nevertheless, the fluorescence quenching mechanism, which leads to the described observations, is not known for ζ_{m} and was subject of this study.

The quantum chemical calculations for ζ_{m} revealed the existence of a dark doublet state (D_1), which has no equivalent in $\zeta_{\text{m}}^{\text{f}}$. Thus, the dark D_1 -state plays the key role in the quenching mechanism of ζ_{m} . The remaining question is, how the D_1 -state is populated. Furthermore, it has to be clarified if the fluorescence quenching *via* the D_1 -state is the only possible quenching pathway. To answer these questions, it is necessary to compare the properties of both labels in detail. The basis of this comparison is, that both labels contain basically the same chromophoric moiety.

Characterization of $\zeta_{\text{m}}^{\text{f}}$. For $\zeta_{\text{m}}^{\text{f}}$ quantum-chemical calculations show that it exhibits a singlet ground state (S_0) and that an excitation into the first excited singlet state (S_1) is allowed. Although the energies of the S_1 - and T_3 -state are almost identical, ISC into higher excited triplet states is unlikely since both the S_1 - and the T_3 -state, correspond to $\pi-\pi^*$ excitations.

Fluorescence-lifetime measurements of $\zeta_{\text{m}}^{\text{f}}$ reveal two ns-decay-components. The longer ns-component (τ_1) dominates the signal decay in polar, aprotic and protic solvents with a high ability of intermolecular interactions while in nonpolar, aprotic solvents with a low ability of intermolecular interactions, the contributions of the two ns-components (τ_1 , τ_2) to the decay are similar to each other. Accordingly, τ_1 is affected by specific and nonspecific solvent interactions, while τ_2 is only affected by nonspecific solvent interactions. The two decay components indicate at least two separate excited states or conformations of the molecule. One of the states is represented by τ_1 and the other state is represented by τ_2 . However, a clear assignment of these states was not possible. The population of a locally excited and a charge transfer state could be excluded by quantum-chemical calculations. Apart from this mechanism, the biexponential fluorescence decay could be explained by two conformations of

the molecule.¹⁷ But in this case one would expect – at least slightly – different emission spectra for both conformations, which was not observed for $\zeta_{\text{m}}^{\text{f}}$. Thus, both states can only be distinguished by specific solvent interactions. Hence, the observed H/D-effect for $\zeta_{\text{m}}^{\text{f}}$ has to be due to H-bond formation (de-/protonation) of chromophore (e.g. on N3) and solvent molecules (cf. Fig. 1a). Obviously, this changes the stabilization and population of the state with the τ_1 lifetime. A clear assignment of τ_1 and τ_2 for $\zeta_{\text{m}}^{\text{f}}$ was not possible. However, τ_1 is the dominating component and τ_2 can be ignored – at least in aqueous solutions of the chromophore – because of its small contribution to the overall fluorescence decay. Thus for simplification we only discuss τ_1 as the lifetime of the solvent relaxed S_1 -state (solvation induced stabilization; $S_{1\text{solv}}$).

Model for $\zeta_{\text{m}}^{\text{f}}$ photodynamics. We propose the following model for the photodynamics of $\zeta_{\text{m}}^{\text{f}}$ in aqueous buffer: $\zeta_{\text{m}}^{\text{f}}$ is excited from the ground state S_0 at the Franck–Condon (FC) point to the S_1 -state potential-energy surface (Fig. 6a). From the FC point, $\zeta_{\text{m}}^{\text{f}}$ undergoes an internal relaxation into the lowest vibrational level of the S_1 -state (TAS: 1.4 ps). The S_1 -state interacts with the solvent (solvent relaxation, TAS: about 200 ps; TCSPC: 300 ps). The $S_{1\text{solv}}$ -state is depopulated radiatively and non-radiatively, into the S_0 (TCSPC and TAS: 4.1 ns).

Model for ζ_{m} photodynamics. Furthermore, we propose a different model for the photodynamics of ζ_{m} in aqueous buffer: ζ_{m} is in a doublet ground state (D_0) and excitation into its excited (D_1) state is dipole-forbidden, since it corresponds to an $n\text{--}\pi^*$ excitation, whereas D_2 -excitation is allowed. Analogous to $\zeta_{\text{m}}^{\text{f}}$, the energies of the D_2 - and the Q_4 -state (Table S2, ESI†) are almost identical, but ISC from the D_2 -state into excited quartet states is not possible, because it would violate angular momentum conservation (El-Sayed rule).⁷⁷ ISC from D_1 -state into Q_n -state, however, is allowed. Note that the S_1 -state of $\zeta_{\text{m}}^{\text{f}}$ corresponds energetically to the D_2 -state of ζ_{m} (Tables 1 and 2), which consolidates the experimental steady-state absorption and fluorescence spectra of both compounds with the theoretical predictions.

The D_1 -state. The main difference between both compounds, as stated above, lies in the existence of the D_1 -state in ζ_{m} , which

has no directly corresponding state in $\zeta_{\text{m}}^{\text{f}}$. D_1 is a spectroscopically dark state. The calculated energy gap between D_2 - and D_1 -state is small (0.3 eV at SF-TDDFT level). Thus, according to the energy gap law,^{78,79} the D_1 -state is likely populated *via* a fast IC from the D_2 -state, which quenches the $D_2 \rightarrow D_0$ fluorescence (Fig. 6b).

The difference densities show for the D_1 -state a localized electron density at the nitroxide group of ζ_{m} (Table 2). But the nitroxide group shows no noteworthy absorption in the observed UV/vis region. Thus, it is neither possible to detect the D_1 -state *via* UV-vis spectroscopy, nor to determine the lifetime of this state. Presumably, the D_1 -state is depopulated *via* IC into the D_0 -state or *via* ISC into a higher quartet state Q_n . As can be seen in the time-slices (Fig. S6b and S9, ESI†), there is a small remaining ESA signal at the end of the measurement window (1.5 ns) that is slightly shifted to longer wavelengths. This remaining ESA, with a spectral maximum around 460 nm, might be due to the $D_1 \rightarrow D_n$ absorption (see Table 1 and Table S2, ESI†) or a quartet absorption.

Comparison with other quenching mechanisms. To verify this quenching model, featuring an IC into a dark excited doublet state, we compared our spectroscopic and theoretical data with other possible quenching pathways.

ISC. One typical quenching pathway would be an enhanced ISC. But, as stated above, the quantum chemical calculations show that ISC is unlikely according to the El-Sayed rules.⁷⁷ Nevertheless, there is a detectable phosphorescence decay for ζ_{m} . This might be due to the continuous wave excitation at 77 K in the experiment. Under such conditions, a weak population of quartet states *via* ISC cannot be ruled out completely.

Charge- or proton-transfer. The strong fluorescence quenching in ζ_{m} in comparison to $\zeta_{\text{m}}^{\text{f}}$ could also be due to a charge-transfer or a proton-transfer process.²⁸ However, an efficient quenching can be seen in all the solvents that were tested, independent of solvent polarity or proticity.

In particular, the fluorescence decays of ζ_{m} in H_2O and D_2O only differ in the ns-components (τ_1 , τ_2). The fast and strong ps-decay component (τ_3) present in ζ_{m} was not affected by $\text{H}_2\text{O}/\text{D}_2\text{O}$ exchange. Altogether, a charge-transfer process can be excluded as a main fluorescence-quenching pathway for ζ_{m} , too. This was also confirmed by quantum chemical calculations.

TD-DFT calculation showed no indication for a charge transfer-like doublet state (D_1 - or D_2 -state).

Nevertheless there is noticeable solvent dependence of the average fluorescence lifetime, especially of the weak ns components (τ_1 , τ_2), as already described for $\zeta_{\text{m}}^{\text{f}}$. Thus, there are at least two separate depopulation pathways for the D_2 -state of ζ_{m} . One pathway leads to efficient fluorescence quenching, the other pathway leads to two less populated emitting excited states, resembling the situation in $\zeta_{\text{m}}^{\text{f}}$.

In the present study, ζ_{m} was treated as a molecular unit (one quantum system) which leads to electronic states with doublet or quartet multiplicity. In this case it was possible to exclude EISC, charge transfer and proton transfer or exchange as possible quenching mechanisms.

The separated moiety picture. Because the difference densities of the D_2 - and D_1 -state have virtually no overlap and are separately

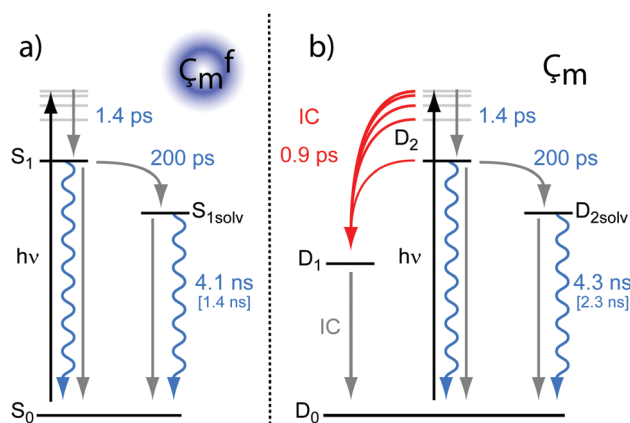


Fig. 6 Photodynamics of $\zeta_{\text{m}}^{\text{f}}$ (a) and ζ_{m} (b). Radiative and non-radiative transitions are displayed as blue wavy and grey (red) regular arrows, respectively.

localized on the chromophore (D_2/S_1 -state) or nitroxide (D_1 -state) moiety one is tempted to discuss ζ_m as two electronically independent but structurally linked moieties (Table 2). In this case the chromophore moiety could be seen as an independent singlet and the nitroxide moiety as an independent doublet. Excitation energy transfer between these moieties, may then be discussed in the picture of Förster and Dexter transfer.

In Table 2 it can be seen that for the D_1 -state, there are only difference densities on the nitroxide moiety. For the D_2 -state, on the other hand, only difference densities on the chromophore moiety are present. Thus, when the molecule is transferred from D_2 - to D_1 -state there are only two different ways to account for these difference charge densities:

(1) An electron of the excited chromophore moiety relaxes from D_2 to the ground state of the chromophore, while an electron of the ground state nitroxide moiety is excited in the D_1 -state of the same moiety. This would resemble a Förster mechanism.

(2) An electron is moved from the excited chromophore moiety of the D_2 -state directly to the lowest unoccupied orbital located at the nitroxide moiety. At the same time an electron of the highest occupied nitroxide orbital has to be transferred to the ground state of the chromophore moiety. This would correspond to the formation of the D_1 -state by a Dexter mechanism.

Förster mechanism. Accordingly, excitation energy transfer from the D_2 -state of the nitroxide *via* Förster resonance energy transfer to the D_1 -state of the chromophore would also be a possible quenching mechanism. Nevertheless, the nitroxide group itself does not affect the steady-state absorption spectrum of ζ_m , *i.e.* has no transition dipole moment.⁴⁶ Accordingly, as discussed for many other similar compounds,^{31,34,35,40,41,44,48,80,81} energy transfer *via* a Förster mechanism between the chromophore moiety (donor) and the nitroxide moiety (acceptor) can be excluded as relevant fluorescence quenching mechanism.⁴⁰

Dexter mechanism. For Dexter energy transfer or electron exchange on the other hand a distance between the chromophore (donor) and the nitroxide (acceptor) smaller than 1 nm is needed, which is within ζ_m clearly given through the direct linkage of both moieties. Due to the additionally small energy difference between the D_2 - and D_1 -states, Dexter energy transfer may thus be one way to interpret the non-radiative decay of the molecule from the D_2 -state into the D_1 -state.

Summary. In summary, since alternative quenching mechanisms, like FT, EISC and ET, do not comply with the spectroscopic and theoretical data, fast non-radiative IC from the initially excited D_2 - *via* the dark D_1 - back to D_0 -state is the most probable fluorescence quenching channel in ζ_m , which can be interpreted as an intramolecular Dexter energy transfer from the chromophore to the nitroxide.

Conclusion

In this article, a detailed steady-state and time-resolved spectroscopic characterization of ζ_m and ζ_m^f in solution is presented. We show fluorescence and absorption spectroscopy as well as quantum-chemical calculations of these nucleoside monomers.

We find that intermolecular interactions directly affect the fluorescence lifetime and the relative quantum yield of ζ_m^f . Therefore, ζ_m^f is a sensitive probe for its microenvironment and a promising RNA fluorescence label. Moreover, in the course of the spectroscopic characterization of ζ_m , a strong intramolecular fluorescence-quenching effect could be shown and quantified. Its mechanism is identified as rapid internal conversion from D_2 - into the dark D_1 -state. The D_1 -state is depopulated into the ground state *via* another IC and into higher quartet states *via* ISC. The dark D_1 -state, which is an intermediate in the course of the fluorescence quenching, is quite remarkable, because the usually proposed enhanced internal conversion leads directly into the doublet ground state. This mechanism can be interpreted as an intramolecular Dexter energy transfer from the chromophore moiety to the nitroxide moiety.

While, the findings on the quenching mechanism of ζ_m represent a substantial contribution to the field of the long-debated nitroxide fluorophores, the understanding of the photodynamics of ζ_m^f will help to interpret and understand results of experiments with ζ_m^f as fluorescent RNA label. Thus, the presented data will be useful as a landmark in further studies with these versatile spectroscopic labels.

In fact, first studies with ζ_m^f labelled RNA single and double strands confirm the favorable photophysical properties and the here proposed microenvironmental sensitivity. Detailed experiments with ζ_m^f as reporter group for RNA-dynamics are currently underway.

Conflicts of interest

There are no conflicts to declare.

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