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Distinguishing Förster Resonance Energy Transfer and Solvent-Mediated Charge-Transfer Relaxation Dynamics in a Zinc(II) Indicator: A Femtosecond Time-Resolved Transient Absorption Spectroscopic Study

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A bifluorophoric molecule (**1**) capable of intramolecular Förster Resonance Energy Transfer (FRET) is reported. The emission intensity of the FRET acceptor in **1** depends on the molar absorptivity of the donor, which is a function of zinc(II) complexation. The FRET dynamics of [Zn(**1**)](ClO₄)₂ is characterized by femtosecond time-resolved transient absorption spectroscopy. The solvent-mediated relaxation of the charge-transfer (CT) state of the isolated donor and the FRET process of the donor-acceptor conjugate are on similar time scales (40–50 ps in CH₃CN), but distinguishable by the opposite solvent polarity dependency. As the solvent polarity increases, the efficiency of Coulombic-based FRET is reduced, whereas CT relaxation is accelerated. In addition to revealing a method to distinguish CT and FRET dynamics, this work provides a photophysical foundation for developing indicators based on the FRET strategy.

We outline a strategy of developing fluorescent indicators for zinc(II) ions based on Förster Resonance Energy Transfer (FRET).¹ This paper focuses on the characterization of an intramolecular FRET process in a zinc(II) complex by femtosecond time-resolved transient absorption spectroscopy. In particular, a method is reported to distinguish FRET and solvent-mediated relaxation of charge-transfer (CT) state of the FRET donor that have similar time constants. The utility of the indicators developed based on this strategy in fluorescence microscopic imaging will be described in a later report.

The rate of FRET (k_{FRET}) as represented in the Förster formulism (eq. 1)² is a function of the distance between the energy donor and acceptor (r), the spectral overlap integral between the donor emission and the acceptor absorption spectra ($J(\lambda)$), the orientation factor (κ) associated with the dipole-dipole interaction between the donor and the acceptor,³ the fluorescence quantum yield of the donor in the absence of an acceptor (ϕ_D), and the refractive index of the medium (η), which relates to dielectric constant ϵ as shown in eq. 2. The efficiency of FRET (E) can be determined quantitatively from time-domain measurements (eq. 3), where τ_D is the lifetime of the

donor in the absence of an acceptor, and τ_{DA} is the time constant for energy transfer in the donor-acceptor complex. As a ramification of the Förster formulism, changing the donor-acceptor distance (r)^{4,5} or the spectral overlap integral ($J(\lambda)$)^{6–8} via analyte binding has been employed in developing fluorescent indicators. Herein, we provide a strategy for an analyte to trigger the enhancement of the acceptor fluorescence, when the values of r and $J(\lambda)$ are relatively insensitive to analyte binding. The ultrafast FRET dynamics is characterized by femtosecond time-resolved transient absorption spectroscopy, in which a solvent polarity dependency is shown to distinguish the dynamics of CT and FRET on similar time scales.

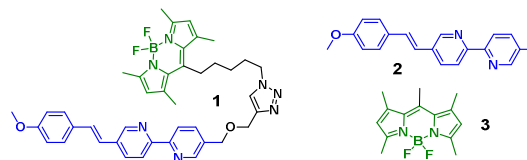
$$k_{FRET} = constant \cdot \frac{\kappa^2 J(\lambda) \phi_D}{r^6 \eta^4 \tau_D} \quad (1)$$

$$\eta^2 = \epsilon \quad (2)$$

$$E = 1 - \frac{\tau_{DA}}{\tau_D} \quad (3)$$

When a CT-type fluorophore is selected as the FRET donor in a bifluorophoric molecule, the acceptor emission intensity at a fixed excitation wavelength may become a function of the concentration of an analyte that alters the absorption and/or emission of the donor upon formation of a complex. For example, if the analyte binding increases the molar absorptivity of the donor at the excitation wavelength while r and $J(\lambda)$ are barely altered, the acceptor emission is expected to increase as a function of the analyte concentration.

Chart 1. Structures



Compound **1** (Chart 1) is reported herein as an example to illustrate the aforementioned strategy. This compound is a conjugate of zinc(II)-binding arylvinylbipyridyl FRET donor moiety (blue) and

BODIPY fluorophore as the FRET acceptor (green). Compound **2** is the isolated FRET donor of the CT type, whose absorption and emission spectra shift bathochromically upon zinc(II) complex formation.⁹ Compound **3** is the isolated FRET acceptor BODIPY.¹⁰ The synthesis of compound **1** is included in the Supporting Information (SI).

The normalized absorption spectrum of FRET donor **2** is shown in Fig. 1 (brown line). The molar absorptivity of **2** at 405 nm, which is the excitation wavelength of the steady state experiments, is $7.3 \times 10^2 \text{ M}^{-1} \text{ cm}^{-1}$. Upon binding zinc(II) to form $[\text{Zn}(\mathbf{2})](\text{ClO}_4)_2$ in CH_3CN , the bathochromically shifted spectrum (blue line in Fig. 1) increased the molar absorptivity at 405 nm to $1.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, a 25-fold enhancement. The emission spectrum of $[\text{Zn}(\mathbf{2})](\text{ClO}_4)_2$ (green line) overlapped well with the absorption spectrum of FRET acceptor **3** (red line), which suggested that efficient intramolecular FRET in the donor-acceptor conjugate **1** should be possible.

The spectral overlap integrals $J(\lambda)$ of the intramolecular FRET pair in **1** and its zinc(II) complex have close values of $4.6 \times 10^{14} \text{ nm}^4 \text{ M}^{-1} \text{ cm}^{-1}$ and $6.1 \times 10^{14} \text{ nm}^4 \text{ M}^{-1} \text{ cm}^{-1}$, respectively.¹¹ Furthermore, the donor-acceptor distance (r) is not expected to change upon zinc(II) coordination at the donor moiety. The fluorescence intensity of the acceptor, therefore, shall primarily be a function of the zinc(II)-dependent excitation of the donor. We postulated that the donor-acceptor conjugate **1** upon zinc(II) coordination result in the enhancement of BODIPY (acceptor) fluorescence, due to the zinc(II)-dependent increase of molar absorptivity at the excitation wavelength (405 nm).

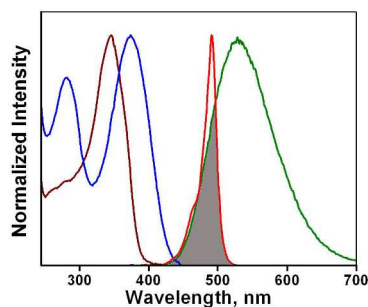


Fig. 1 Normalized absorption spectra of **2** (brown), $[\text{Zn}(\mathbf{2})](\text{ClO}_4)_2$ (blue), **3** (red), and emission spectrum of $[\text{Zn}(\mathbf{2})](\text{ClO}_4)_2$ (green). The shaded area represents the spectral overlap between the emission of $[\text{Zn}(\mathbf{2})](\text{ClO}_4)_2$ (donor) and absorption of **3** (acceptor).

To test the hypothesis that zinc(II) binding to the donor increases the acceptor emission intensity in **1**, steady-state absorption and emission spectra were acquired in CH_3CN . The absorption spectrum of **1** beyond 300 nm (Fig. 2a) included two bands that centered at 347 nm ($\epsilon = 3.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) and 494 nm ($\epsilon = 5.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$), which were assigned to the absorption maxima of the FRET donor and acceptor moieties, respectively. These two bands coincided with those of the isolated donor (**2**) and acceptor (**3**) molecules, suggesting little interaction between the two fluorophores in **1** in the ground state. Upon increasing the concentration of $\text{Zn}(\text{ClO}_4)_2$, the FRET donor absorption band maximum shifted from 347 nm to 376 nm, while the FRET acceptor band remained largely unchanged. Consequently, upon excitation of the zinc(II)-bound donor moiety of **1** at 405 nm, the emission intensity of the acceptor increased in a zinc(II)-dependent manner (Fig. 2b). Under saturation conditions, the fluorescence quantum yield of $[\text{Zn}(\mathbf{1})](\text{ClO}_4)_2$ was measured at 0.39, while that of $[\text{Zn}(\mathbf{2})](\text{ClO}_4)_2$ was 0.46 in CH_3CN .

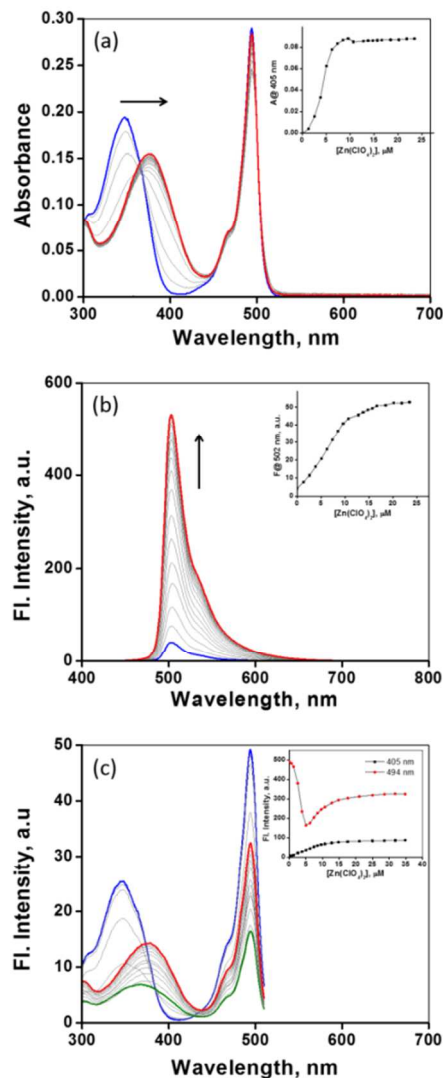


Fig. 2 (a) Absorption spectra of compound **1** (5.0 μM) in CH_3CN as a function of $[\text{Zn}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}]$. (b) Corresponding changes in the emission spectrum at $\lambda_{\text{ex}} = 405 \text{ nm}$. (c) Corresponding changes in the excitation spectra at $\lambda_{\text{em}} = 520 \text{ nm}$. Blue, red, and green highlight the initial, final, and intermediate spectra, respectively. Inset shows the emission intensity vs zinc(II) concentration at selected wavelengths.

The zinc(II)-dependent excitation spectral shift of compound **1** (Fig. 2c) mirrored in large part that of the absorption spectrum. Therefore, the emission of **1** at 520 nm resulted from either direct excitation of BODIPY acceptor moiety (494 nm band), or sensitized excitation of BODIPY via FRET from the zinc(II)-bound arylvinylbipyridyl (378 nm band). The initial addition of $\text{Zn}(\text{ClO}_4)_2$ quenched the fluorescence (from blue to green traces; see the data at 494 nm in the inset), attributable to a relatively low quantum yield of the 2:1 (ligand/zinc(II)) complex.^{9a} The fluorescence intensity was partially recovered upon further addition of $\text{Zn}(\text{ClO}_4)_2$ (from green to red traces in Fig. 2c) as the 1:1 (ligand/zinc(II)) complex was forming.

The excited state dynamics of zinc(II) complexes of **1** and **2** were studied using femtosecond time-resolved transient absorption (TA) spectroscopy. The femtosecond TA set-up was described in detail as SI and in a previous publication.¹² The average time constants were tabulated with their standard deviations in Table 1. A solution of $[\text{Zn}(\mathbf{1})](\text{ClO}_4)_2$ was excited at 400 nm, and two TA bands were

observed (Fig. 3a). The band centering at 560 nm was assigned to the TA of the zinc(II)/donor complex, while the 500-nm band was attributed to the BODIPY acceptor, based on the comparisons with the TA spectra of the isolated donor $[\text{Zn}(2)](\text{ClO}_4)_2$ (blue in Fig. 3c) and acceptor **3** (red). Two components were observed in the temporal evolution of the donor band of $[\text{Zn}(1)](\text{ClO}_4)_2$ at 560 nm (Fig. 3b, blue). τ_1 (2.8 ps) was attributed to the internal conversion, and τ_2 (41 ps) was assigned to FRET. The change of the acceptor band at 500 nm contained three components (Fig. 3b, red): τ_1 at 7.4 ps was internal conversion of the directly excited BODIPY; τ_2 (42 ps), which was a growth, was attributed to FRET, based on its correlation to the donor τ_2 decay at 560 nm. A much longer τ_3 (3.2 ns) agreed well with the fluorescence lifetime of $[\text{Zn}(1)](\text{ClO}_4)_2$ (4.0 ns), determined using nanosecond time-correlated single photon counting (TCSPC) technique (SI).

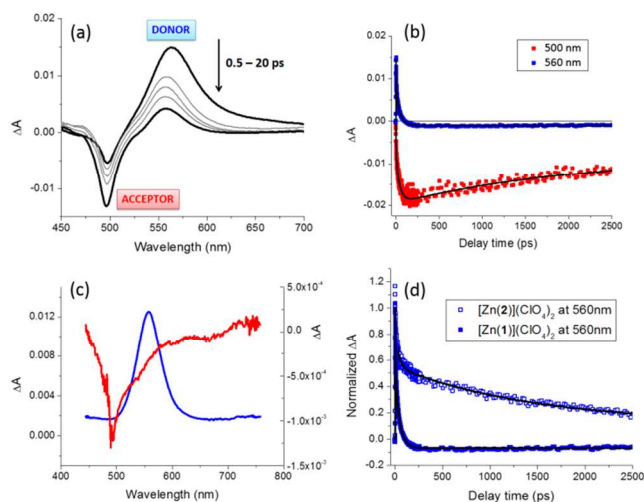


Fig. 3 (a) Transient absorption (TA) spectra (0.5–20 ps) of complex $[\text{Zn}(1)](\text{ClO}_4)_2$ (0.1 mM) in CH_3CN , excited at 400 nm. (b) The changes of the TA at 500 nm (■, red) and 560 nm (■, blue). (c) The TA spectra of the isolated donor $[\text{Zn}(2)](\text{ClO}_4)_2$ (blue, 2 ps), and the isolated acceptor **3** (red, 1 ps) in CH_3CN . (d) The TA decays at 560 nm of $[\text{Zn}(1)](\text{ClO}_4)_2$ (■, blue) and $[\text{Zn}(2)](\text{ClO}_4)_2$ (□, blue). An excitation pulse energy of 400 nJ/pulse was used for all experiments.

For the isolated FRET donor $[\text{Zn}(2)](\text{ClO}_4)_2$, only the 560 nm band was observed (blue in Fig. 3c), the decay of which consisted of three components: τ_1 of 2.8 ps as internal conversion, τ_2 of 53 ps, and τ_3 of 1.5 ns, which was assigned to fluorescence decay.^{7,9a} Based on the CT nature of compound **2**,^{9a} τ_2 may be assigned to the solvent relaxation of the CT excited state. The similarity in the time constants of the FRET process in $[\text{Zn}(1)](\text{ClO}_4)_2$ and the solvent-mediated CT relaxation in $[\text{Zn}(2)](\text{ClO}_4)_2$ prompted us to examine the solvent dependencies of these two rates.

The TA data of $[\text{Zn}(1)](\text{ClO}_4)_2$ and $[\text{Zn}(2)](\text{ClO}_4)_2$ were collected in several solvents that cover a range of dielectric constants spanning from $\epsilon = 4.8$ to $\epsilon = 38$. The time constants (τ_2) that were assigned to either FRET in $[\text{Zn}(1)](\text{ClO}_4)_2$ or solvent relaxation of CT in $[\text{Zn}(2)](\text{ClO}_4)_2$ were listed in Table 2. The τ_2 value of the isolated FRET donor $[\text{Zn}(2)](\text{ClO}_4)_2$ decreased as the solvent polarity increased. This observation is consistent with the property of a CT fluorophore, which undergoes a faster solvent relaxation in a more polar solvent.^{13,14} The τ_2 value in the donor-acceptor conjugate $[\text{Zn}(1)](\text{ClO}_4)_2$ had the opposite trend with solvent polarity. This behavior is a direct ramification of the Förster formalism (eq. 1), of which the dipole/dipole interaction that leads to resonance energy transfer is attenuated as the polarity of the solvent increases. Given

the relative insensitivity of fluorescence quantum yield of the donor (ϕ_D) and the spectral overlap integral ($J(\lambda)$) to solvent polarity (Table 2), the approximate linear correlation ($R^2 = 0.78$) between τ_2 at 500 nm (τ_{growth}) and $\tau_D \epsilon^2$ shown in Fig. 4 offers a rare confirmation of the solvent dependency of Förster-type energy transfer.

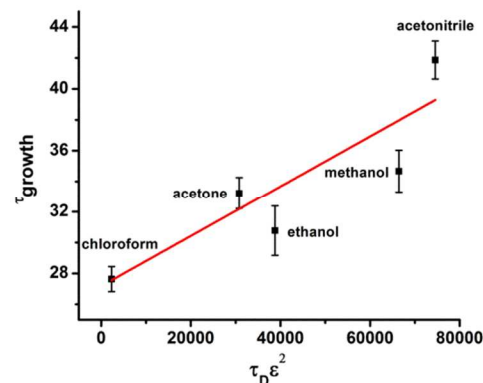


Fig. 4 The correlation between FRET time constant (τ_2 at 500 nm of $[\text{Zn}(1)](\text{ClO}_4)_2$ - τ_{growth}) and solvent polarity (ϵ^2). τ_D values were determined from measurements on the isolated donor $[\text{Zn}(2)](\text{ClO}_4)_2$. τ_{growth} was obtained by analyzing the magnitude of the 500-nm signal of the $[\text{Zn}(1)](\text{ClO}_4)_2$ complex as a function of pump-probe delay time.

Based on the data from femtosecond TA experiments, a model of the excited state dynamics of zinc(II) complexes of **1** and **2** in CH_3CN was constructed (Fig. 5). When $[\text{Zn}(1)](\text{ClO}_4)_2$ was excited (pumped) at 400 nm where the FRET donor absorbed, after a rapid internal conversion ($\tau_1 = 2.8$ ps), FRET ($\tau_2 = 41$ ps) populated the S_1 of the acceptor which absorbed at 500 nm. This band decayed at a rate ($\tau_3 = 3.3$ ns) comparable to the fluorescence lifetime of the acceptor (SI). For the FRET donor $[\text{Zn}(2)](\text{ClO}_4)_2$, after excitation at 400 nm, the transient band decayed with three time constants. In addition to internal conversion (2.8 ps), τ_2 (53 ps) was assigned to the solvent relaxation of the charge transfer (CT) state, and τ_3 (1.5 ns) was attributed to the emission of the CT state. Both FRET and CT solvent relaxation processes had comparable rates; but they were distinguished by the opposite solvent dependency – FRET efficiency was decreased while CT was accelerated as the solvent polarity increased.

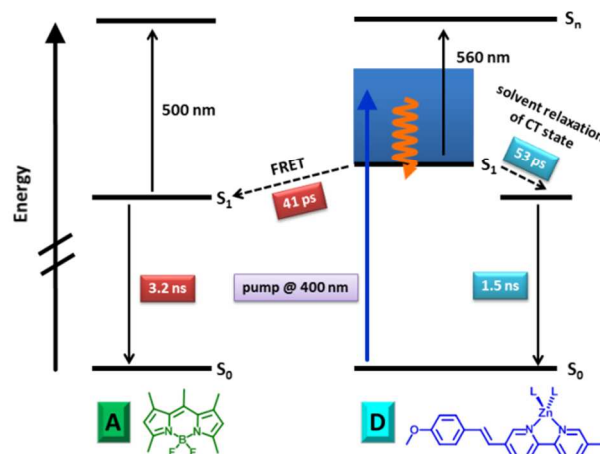


Fig. 5 Schematics of key excited-state decay pathways of the FRET conjugate $[\text{Zn}(1)](\text{ClO}_4)_2$ (left, in red) and isolated donor $[\text{Zn}(2)](\text{ClO}_4)_2$ (right, in blue).

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Table 1. Transient decay time constants excited at 400 nm in CH₃CN.

compound	τ_1 (amplitude) @ 560 nm	τ_2 (amplitude) @ 560 nm	τ_3 (amplitude) @ 560 nm	τ_1 (amplitude) @ 500 nm	τ_2 (amplitude) @ 500 nm	τ_3 (amplitude) @ 500 nm
[Zn(2)](ClO ₄) ₂	2.83±1.49 ps (0.17)	53.4±14.6 ps (0.21)	1.52±0.03 ns (0.49)	-	-	-
[Zn(1)](ClO ₄) ₂	2.85±0.23 ps (0.21)	40.8±0.2 ps (0.60)	-	7.39±0.55 ps (0.12)	41.9±1.2 ps (0.32)	3.29±0.91 ns (-0.42)

Table 2. Solvent polarity dependency of the time constants (τ_2 , ps) of CT solvent relaxation of [Zn(2)](ClO₄)₂ at 560 nm, and FRET of [Zn(1)](ClO₄)₂ at 560 and 500 nm.

compound	wavelength	chloroform	acetone	ethanol	methanol	acetonitrile
[Zn(1)](ClO ₄) ₂	500 nm	27.6±0.8	33.2±1.0	30.8±1.6	34.7±1.4	41.9±1.2
[Zn(1)](ClO ₄) ₂	560 nm	35.3±0.3	35.0±0.4	33.4±2.5	39.9±0.5	40.8±0.2
[Zn(2)](ClO ₄) ₂	560 nm	100.7±4.8	69.8±3.2	64.3±14.1	61±7.6	53.4±14.6
Dielectric constant (ϵ)		4.8	21	25	33	38
$J(\lambda)$ of [Zn(2)](ClO ₄) ₂ and 3 (10^{14} nm ⁴ ·M ⁻¹ ·cm ⁻¹)		6.78	6.36	6.54	6.64	6.11
ϕ_D of [Zn(2)](ClO ₄) ₂		0.57	0.52	0.52	0.49	0.46
ϕ_{FRET} of [Zn(1)](ClO ₄) ₂		72%	52%	52%	43%	21%

Conclusions

In summary, compound **1** and its zinc(II) complexes exhibited efficient intramolecular FRET. Rather than altering the distance or spectral overlap integral between the donor and the acceptor, zinc(II) coordination at the FRET donor moiety of **1** increased the molar absorptivity of the donor at the excitation wavelength (405 nm), thus enhancing the acceptor emission intensity. The ultrafast FRET process (41 ps in CH₃CN) was characterized by femtosecond time-resolved transient absorption spectroscopy, which also revealed the opposite solvent dependencies of the FRET and CT solvent relaxation dynamics. Resonance energy transfer was hindered, while solvent relaxation of the CT state was facilitated as solvent polarity increased. This observation was consistent with the theories of FRET and charge transfer, which provided a straightforward method to distinguish the two competitive relaxation processes of comparable rates. Both FRET and CT type fluorophores would result in ratiometric fluorescence readout when used in indicators.¹⁵⁻¹⁷ Importantly, these data confirmed intramolecular resonance energy transfer as the mechanism for zinc(II)-dependent fluorescence of **1**, a conclusion that is employed in our laboratory for developing zinc(II) indicators.

Acknowledgment

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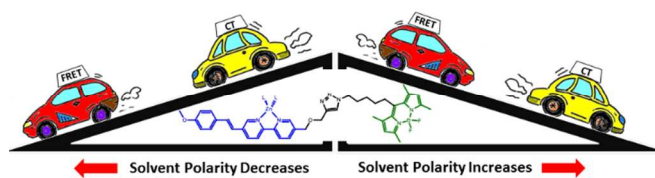
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Graphic Abstract



The rates of intramolecular Förster resonance energy transfer and solvent-mediated relaxation of charge-transfer excited state have opposite solvent polarity dependencies.