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Journal Article

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Publication date:

2019-11-07

Permanent link:

<https://doi.org/10.3929/ethz-b-000379975>

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Originally published in:

The Journal of Physical Chemistry Letters 10(21), <https://doi.org/10.1021/acs.jpclett.9b02621>

Funding acknowledgement:

169057 - Generation of spin-label based restraints on biomolecular structure and their use in hybrid structure modelling (SNF)

Linear and Kinked Oligo(phenyleneethynylene)s as Ideal Molecular Calibrants for Förster Resonance Energy Transfer

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ABSTRACT: We show that oligo(phenyleneethynylene)s (oligoPEs) are ideal spacers for calibrating dye pairs used for Förster resonance energy transfer (FRET). Ensemble FRET measurements on linear and kinked diads with such spacers show the expected distance- and orientation-dependence of FRET. Measured FRET efficiencies match excellently with those predicted using a harmonic segmented chain model, which was validated by end-to-end distance distributions obtained from pulsed electron paramagnetic resonance measurements on spin-labeled oligoPEs with comparable label distances.

Förster resonance energy transfer (FRET)^{1,2} experiments exploit the distance-dependence of dipolar coupling between an electronically excited fluorescent dye, acting as energy donor, and a chromophore in its ground state, acting as energy acceptor. Since FRET provides a means for sensing inter- and intramolecular distances in the range of 30–100 Å, many FRET-based techniques have emerged,^{3–9} e.g., in studies of protein folding and protein interaction.^{10–13}

Förster's theory¹ relates the FRET efficiency E with the inter-dye distance r_{da} according to:

$$E = \frac{1}{1 + \left(\frac{r_{da}}{R_0}\right)^6}, \quad (1)$$

where R_0 is the critical Förster distance, at which $E = 0.5$. R_0 depends on the spectroscopic properties of the dyes, their environment, and their relative orientation:

$$R_0^6 = c\kappa^2 Q_D / n_m^{-4}. \quad (2)$$

Here, c is a collection of constants, Q_D the fluorescence quantum yield of the donor, J the spectral overlap integral between donor emission and acceptor absorption, and n_m the refractive index of the medium. The orientation factor κ^2 , with $0 < \kappa^2 < 4$, describes the relative orientation of the donor and acceptor transition dipole moments:

$$\kappa^2 = (\cos\theta_T - 3\cos\theta_D \cos\theta_A)^2, \quad (3)$$

where θ_D and θ_A are the angles between the individual transition moments and the inter-dye displacement vector, and θ_T is the angle between the two transition moments. R_0 is determined with measurements on the bare dyes and is usually calculated assuming an isotropic, dynamically averaged ensemble of orientations, resulting in $\langle\kappa^2\rangle = 2/3$.

Although FRET is commonly employed as a “spectroscopic ruler,”¹⁴ difficulties remain in translating FRET efficiencies into distances. Deviations from the expected scaling of E with r_{da} can occur at short distances (<30 Å), since the point-dipole approximation no longer holds,^{15–17} and other energy transfer processes dominate.¹⁸ Incomplete orientational averaging can also confound distance determination.^{19–22} Prior efforts to quantify the distance- and orientation-dependence in FRET have used dye-tagged oligoprolines^{14,23–27} and DNA duplexes.^{28–37} However, owing to the conformational diversity of such compounds, and also specific electronic reasons, experimentally determined distances usually deviate from those derived from even advanced molecular dynamics simulations. This is in part because, even when great caution is exercised, errors in experimental distances are at least

Chart 1. Bare dyes, FRET diads, and spin-labeled oligoPEs. For further details, see SI.

*“R” denotes either *n*-hexyl or 6-methoxyhexyl substituents.

7%.³⁸ Furthermore, the orientation effect is difficult to study with such systems: the relative orientation of the dyes is hard to control in oligoprolines; and in DNA systems the range of accessible κ^2 values is rather narrow. Thus, systematic studies with conformationally unambiguous molecules would bring significant value, not only from a fundamental perspective, but also for comparing structural information obtained from FRET with other experiments,^{39–41} and for the validation of modern computational methods.^{42–45}

Figure 1. Fluorescence emission spectra of L_n -DAs (a) and K_n -DAs (b) upon excitation at 450 nm. The spectra are normalized to the acceptor emission maximum (575 nm). Donor fluorescence lifetime of L_n -DAs (c) and K_n -DAs (d) monitored at 525 nm upon excitation at 450 nm (1 channel = 1.2×10^{-11} s), instrument response functions (IRF, black traces), and time-resolved measurements of donor-only conjugates (L_5 -D, K_5 -D, gray traces). Fitted lifetimes are listed in Table S1, and fit residuals are shown in Figure S5.

Here we show with a set of linear (L_n -DA) and kinked (K_n -DA) dye-labeled oligo(phenyleneethynylene)s (oligoPEs) (Charts 1, S1, S2), and an analogous set of linear and kinked spin-labeled oligoPEs, that oligoPEs are ideal spacers for studying the distance- and orientation-dependence of FRET. The dyes used here are perylene monoimide (PMI), which acts as the donor (D), and the aryloxy-substituted variant PMI(OAr)₃, which acts as the acceptor (A). These dyes have high fluorescence quantum yields (>0.85)^{46,47} and exhibit good spectral overlap (Figure S1).⁴⁷ Semi-empirical calculations (Figure S2) show that the transition moment of PMI lies along its long axis, and that of PMI(OAr)₃ deviates by less than 4° from its long axis. Both dyes are connected with the oligoPE such that the relative orientation of their transition moments is essentially rotation-invariant. Disregarding spacer bending, the L_n -DAs are expected to have essentially colinear transition moments and therefore $\kappa^2 \approx 4$, whereas the K_n -DAs should have $\kappa^2 < 4$. The conjugation between the dyes and the PE spacer is essentially broken by the isopropyl substituents at the spacer's terminal benzene rings.⁴⁷ The inter-dye distances of L_n -DAs and K_n -DAs with identical n are, according to a static structural model, very similar (Table S3).

Spectroscopic characterization of the bare dyes PMI and PMI(OAr)₃ proved the suitability of the dye pair. Using the spectroscopic data of the bare dyes and applying eq 2 gives $R_0 = 53 \pm 1$ Å when $\langle \kappa^2 \rangle = 2/3$ (see SI). Measurements on the linear and kinked donor- and acceptor-only conjugates (Chart S1, Figures S3 and S4) show that neither the fluorescence lifetime, nor the emission and absorption profiles over the wavelength range used for FRET (400–700 nm), are appreciably altered upon connecting the dyes with the oligoPEs.

Figures 1a,b compare the fluorescence emission spectra of the L_n -DAs and K_n -DAs. For both, increasing the number n of PE subunits results in a clear enhancement in donor emission (at 525 nm) relative to acceptor emission (at 575 nm), indicating a decrease in E with increasing r_{da} . Furthermore, the spectra suggest that E for the K_n -DAs are systematically lower than for the L_n -DAs with the same n . Thus, either increasing the distance between the dyes, or causing a deviation of their transition moments from colinearity, leads to a reduction in E . Both observations fit well within Förster's framework.

Figures 1c,d compare the results of donor fluorescence lifetime measurements for the L_n -DAs and K_n -DAs ($n = 5, 7, 9$). For comparison, fluorescence decays for donor-only conjugates are overlaid (gray traces). Donor fluorescence lifetimes were extracted from these data using a non-linear least-squares fit and lie in the range 0.2–3.6 ns (Table S1; Figure S5). The donor fluorescence lifetime increases as the number of PE subunits increases, and is always longer in the absence of acceptor (~ 4.2 ns) than in its presence. Furthermore, the donor fluorescence lifetimes for the L_n -DAs are shorter than for the K_n -DAs with the same n . These results agree with the spectral data and show unambiguously that FRET occurs between the two dyes.

FRET efficiencies (Table S1) were determined from donor and acceptor fluorescence emission intensities and from donor fluorescence lifetimes, and show good agreement. Except for the shortest diad **L**₂-**DA**, E lies in the range 0.1–0.9, which encompasses the range typically interpreted in distance determination by FRET.

Experimentally determined FRET efficiencies were related to modeled values for r_{da} , first using a simple static structural model (Figure 2a,b and S6). Experimental efficiencies for the **L**_{*n*}-**DAs** (Figure 2c, colored squares) are systematically higher than for **K**_{*n*}-**DAs** with the same n (colored triangles). This difference can be rationalized in terms of differences in κ^2 . Within the static model, $\kappa^2 = 4$ for the **L**_{*n*}-**DAs**, whereas κ^2 values lie in the range 1.8–2.2 for the **K**_{*n*}-**DAs** (Table S3).

To show that the FRET efficiencies quantitatively conform to expectations, the same data are plotted (Figure 2c, gray markers) against modeled distances corrected for differences in κ^2 . We adopt here the convention of a κ^2 -corrected modeled distance r_{corr} , which is defined in relation to modeled values for r_{da} and κ^2 according to:

$$r_{\text{corr}} = r_{\text{da}} \left(\frac{2/3}{\kappa^2} \right)^{1/6}. \quad (4)$$

On this corrected distance axis, the FRET efficiencies of linear and kinked diads are expected to follow, and indeed do follow, a common curve with an inverse sixth-power dependence on r_{corr} as described by eq 1. A fit to the data with eq 1 (Figure 2c, black trace) yields an R_0 value of

Figure 2. (a) Structure of the donor dye PMI, with the assumed point-dipole position (blue circle) and the calculated direction of its transition dipole moment (blue arrow). The same applies to acceptor dye PMI(OAr)₃ when disregarding the small deviation of the transition dipole moment orientation from colinearity with the long axis of the dye. (b) Geometric definitions for evaluation of modeled κ^2 (eq 3). (c) Measured FRET efficiencies for **L**_{*n*}-**DAs** (squares) and **K**_{*n*}-**DAs** (triangles) as a function of modeled values of r_{da} (colored markers) and r_{corr} (gray markers), using a static model (Figure S6). The curve is a fit to the data using eq 1, which yields $R_0 = 54.1 \pm 0.7$ Å at the 99% confidence level.

54.1 ± 0.7 Å, which agrees with the value determined from steady-state measurements on the bare dyes.

To obtain an estimate of the bending flexibility of the diads, the diads were modeled within the framework of a harmonic segmented chain (HSC) model: the polymer is treated as a chain of segments, and bending occurs only at joints between segments (see SI). The HSC model was developed previously for spin-labeled oligoPEs.^{48,49} Chiefly, harmonic bending potentials for the oligoPE backbone, as well as for the spin labels, were determined using a global fit of end-to-end distance distributions obtained with electron paramagnetic resonance (EPR) spectroscopic measurements on a set of linear spin labeled oligoPEs.

To validate the HSC model, we used herein data obtained with the single frequency technique for refocusing (SIFTER).⁵⁰ SIFTER exploits the fact that the orientation-dependence of the dipole–dipole coupling frequency ω_{dd} in EPR spectroscopy is fully described by the orientation of the molecule with respect to the external magnetic field $\vec{B}_0$.⁵¹

$$\omega_{\text{dd}}(r, \theta) = \frac{1}{r^3} C (1 - 3 \cos^2 \theta), \quad (5)$$

where r is the spin–spin distance and θ is the angle between the spin–spin vector and $\vec{B}_0$. The prefactor $C = \mu_B^2 g_A g_B / \hbar$ includes the Bohr magneton μ_B , the g -values of the two electrons, and the reduced Planck’s

constant $\hbar$. This θ -dependence of ω_{dd} in EPR spectroscopy is analogous to the κ^2 -dependence of E in FRET. However, unlike in FRET, where it is not possible to selectively excite conformers with particular κ^2 values (unless they are immobilized), in EPR spectroscopy, molecules taking on particular θ values can be addressed with specific excitation

Figure 3. (a) Experimental distance distributions obtained with SIFTER on the spin-labeled oligoPEs shown in Chart 1 (black traces), with simulated distributions from an HSC model overlaid (dashed red lines). (b) Comparison of experimentally determined FRET efficiencies to ensemble averages from an HSC model for the **L_n-DAs** (squares) and **K_n-DAs** (triangles). The dashed line corresponds to complete agreement.

frequencies. Detection of only a subset of θ values is called orientation selection,⁵² and can introduce bias in distance measurements for molecules with highly correlated geometries such as the oligoPEs under examination. Here, the excitation frequency was swept over the whole spectrum⁵³ to detect ω_{dd} from spin-labeled oligoPEs of all orientations by SIFTER. The obtained dipolar spectra (Figure S7) match theoretical predictions assuming complete sampling over all θ (and thus orientations).

Figure 3a compares distance distributions for linear and kinked spin-labeled oligoPEs (Chart 1) determined with SIFTER to those generated using an HSC model. Overall, the simulated distributions show good agreement with those measured with respect to their mean distances and shapes, indicating that we can apply these bending potentials to model the FRET diads.

Monte Carlo simulations were used to generate ensembles of diad structures with the HSC model (Figure S8). Modeled average FRET efficiencies were obtained from these ensembles using the R_0 value obtained from steady-state measurements on the bare dyes (53 Å), and show excellent agreement with the measured efficiencies (Figure 3b), for which no knowledge of R_0 is needed.

The results obtained from a simple static picture and those from a more realistic model (which takes bending into account) agree remarkably well (Table S3). This can be understood from the fact that r_{da} and κ^2 are highly correlated in the oligoPEs. Intuitively, in the **L_n-DAs** any backbone bending would serve to decrease both r_{da} and κ^2 ; the former would increase E , while the latter would decrease E . Quantitatively, the HSC model predicts that for the **L_n-DAs** this compensation of effects results in different bending isomers having similar efficiencies (Figure S9). In the **K_n-DAs** this correlation persists, though it is less pronounced.

We note finally that, although MD simulations using a polymer-consistent force field give qualitatively reasonable results (Figure S10), they vastly underestimate the flexibility of shape-persistent polymers of this

size. The agreement between the FRET and EPR data supports the idea that the HSC model more accurately reflects the flexibility of oligoPEs.

In summary, we have shown that oligoPEs are ideal spacers for fundamental studies on FRET. In contrast with the commonly employed oligoproline and DNA FRET spacers, oligoPEs give excellent control over the relative orientation of the dye transition moments. Our results suggest that linear and kinked oligoPEs can be used as calibrants to determine R_0 for any dye pair, without the requirement that the directions of the dye transition moments be known. The dyes of interest can be attached in either a geometrically fixed manner or via a conformationally flexible linker. The latter would enable addressing the issue of orientational averaging for a specific dye pair. OligoPE-based diads could be of significant value for the development of gas-phase energy transfer probes,^{54–58} where for example the direct determination of R_0 by conventional means is not possible, and should be attractive choices for validating computational methods for modeling energy transfer processes, which can now be done at high levels of theory.^{44,45}

ASSOCIATED CONTENT

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ACKNOWLEDGMENTS

This research was supported by Deutsche Forschungsgemeinschaft (GO-555-4/3) (to A.G.) and SNF grant 200020_169057 (to G.J.). We thank Dr. R. Brune for introducing D.S. to the optical measurements and for recording most of the decay traces, and Prof. Dr. M. Sauer for his contributions at the beginning of this project.

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