Measuring distances within unfolded biopolymers using fluorescence resonance energy transfer: The effect of polymer chain dynamics on the observed fluorescence resonance energy transfer efficiency

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Recent years have seen a number of investigations in which distances within unfolded proteins, polypeptides, and other biopolymers are probed via fluorescence resonance energy transfer, a method that relies on the strong distance dependence of energy transfer between a pair of dyes attached to the molecule of interest. In order to interpret the results of such experiments it is commonly assumed that intramolecular diffusion is negligible during the excited state lifetime. Here we explore the conditions under which this “frozen chain” approximation fails, leading to significantly underestimated donor-acceptor distances, and describe a means of correcting for polymer dynamics in order to estimate these distances more accurately. © 2009 American Institute of Physics. [DOI: 10.1063/1.3212602]

I. INTRODUCTION

Single-molecule fluorescence resonance energy transfer (FRET) is a popular method for measuring distances across and conformational rearrangements within biomolecules. The approach relies on energy transfer between a pair of donor (*D*) and acceptor (*A*) fluorescent probes attached to the molecule of interest and takes advantage of the strong dependence of energy transfer on the distance *R* between these dyes. Specifically, an optically excited donor fluorophore can decay back to its the ground electronic state either by re-emitting a photon with a rate $\Gamma = 1/\tau_D^{\text{intr}}$ (where τ_D^{intr} is the intrinsic donor lifetime observed in the absence of energy transfer to the acceptor) or transfer excitation energy to the acceptor with a rate k_{FRET} , resulting in a photon emitted from the acceptor molecule (here we have neglected any other decay mechanisms). The efficiency *E* of FRET (i.e., the fraction of the emitted photons that are emitted by the acceptor) is given by the Förster formula

$$E = \frac{k_{\text{FRET}}}{\Gamma + k_{\text{FRET}}} = \frac{R_0^6}{R^6 + R_0^6}, \quad (1)$$

which results from the power law dependence of the FRET rate k_{FRET}

$$k_{\text{FRET}} = (1/\tau_D^{\text{intr}}) \left(\frac{R_0^6}{R^6} \right). \quad (2)$$

Here R_0 is the Förster radius defined as the distance *R* for which half of the emission is from the acceptor.

Experimental FRET measurements reflect average distances. That is, in practice the instantaneous values of *E* (and accordingly *R*) cannot be measured because the donor-acceptor distance fluctuates on a time scale that is much faster than the time resolution of the experiment.^{1–3} It is commonly assumed that the observed value of the FRET efficiency is equal to the *ensemble average* value of *E*,^{3–12}

$$E_{\text{obs}} = \langle E \rangle = \int_0^\infty dR p(R) \frac{k_{\text{FRET}}(R)}{k_{\text{FRET}}(R) + \Gamma} = \left\langle \frac{R_0^6}{R^6 + R_0^6} \right\rangle, \quad (3)$$

where $p(R)$ is the probability distribution of the donor-acceptor distance. [Note that we use the notation E_{obs} for the FRET efficiency that is *actually observed* to distinguish it from the *instantaneous* value *E*, described by Eq. (1), that would be observed were intrachain dynamic effects negligible.] Equation (3) has been used to estimate the size of unfolded proteins and polypeptides^{4,7,8,11–13} and to estimate the persistence length of single-stranded DNA.⁶

Given a probability distribution $p(R)$, the predicted value of E_{obs} is readily computed from Eq. (3). The inverse task of extracting the polymer's structural properties such as $p(R)$ from the observed FRET efficiency is considerably less trivial and cannot be accomplished without additional assumptions. In order to pursue this, it is common to assume a particular functional form of the distribution $p(R)$ that corresponds to a specific theoretical model of the conformational ensemble in question (e.g., Gaussian chain, Flory's random coil or wormlike chain). One can then extract the parameters of this distribution (e.g., the polymer's persistence length) by fitting the experimental values of E_{obs} using Eq. (3).^{7,13} Of note, recent work by O'Brien *et al.*¹⁴ addresses the sensitivity of this procedure to the particular polymer model chosen.

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Here we discuss a limitation of the above procedure that is, perhaps, even more fundamental than the choice of polymer model employed. Equation (3) is valid under the assumption that a detected photon reports on the instantaneous distance between the donor and the acceptor at the moment the donor is excited. In other words, it assumes that the typical time scale τ_R associated with the fluctuations of the donor-acceptor distance R is much greater than the intrinsic emission lifetime τ_D^{intr} so that R does not change significantly between the moment the donor is excited and time the photon is emitted. As τ_D^{intr} is typically of order of a few nanoseconds,¹⁵ this assumption is likely valid for FRET pairs separated, for example, by rigid, folded biomolecules. In the case of unfolded biopolymers or unstructured regions of folded biopolymers, however, the time scale over which significant conformational reconfiguration occurs may be of similar magnitude to the donor lifetime,¹⁶ thus rendering the assumption that $\tau_R \gg \tau_D^{\text{intr}}$ questionable. If this assumption fails, the observed FRET efficiency will differ from the instantaneous ensemble average predicted by Eq. (3).^{6,17–19} Specifically, E_{obs} will be higher than the value predicted by Eq. (3) and, consequently, the use of Eq. (3) will result in the underestimation of the true mean distance. To see this, imagine that the donor lifetime τ_D^{intr} is very long. After excitation, the donor-acceptor distance will fluctuate significantly before the donor becomes de-excited. According to Eq. (2), it is therefore highly likely that at some instance the distance R will become small enough to enable nearly instantaneous energy transfer to the acceptor. Thus we expect E_{obs} to approach 1 in the limit of $\tau_D^{\text{intr}} \gg \tau_R$ irrespective of the true mean distance.⁶

We note that time resolved fluorescence lifetime measurements provide more information than just the single quantity E_{obs} . Consequently, time resolved data—when available—can be used to constrain the underlying distance distribution $p(R)$ more accurately.^{20,21} Moreover, such experiments can be used to obtain information about chain dynamics.^{18,22} Here, however, we focus on the much more readily measurable FRET efficiency E_{obs} , whose dependence on the time scales of donor emission and polymer reconfiguration has not been quantitatively studied except for the limiting cases of very fast and very slow polymer dynamics.^{23,24} The goal of this paper is thus to analyze quantitatively the errors incurred when polymer dynamics are neglected and the assumptions inherent in Eq. (3) are employed. In the following we derive the exact expression for the observed FRET efficiency E_{obs} independent of the relationship between the time scales of the donor lifetime and polymer dynamics. Of note, the expression we have derived is applicable to any model of end-to-end polymer distance dynamics (e.g., Rouse chain). We then apply this general theory to the case of FRET between the two ends of an unfolded polypeptide in water. Perhaps unexpectedly, we find the difference between E_{obs} and $\langle E \rangle$ to be substantial even when τ_R is almost an order of magnitude larger than τ_D^{intr} . We then invoke scaling arguments to argue that in the limit of long chains E_{obs} should be a universal function of two dimensionless parameters: The ratio $\tau_D^{\text{intr}}/\tau_R$ of the two time scales and the ratio of the length scales, $\sqrt{\langle R^2 \rangle}/R_0$, of the polymer and of

FRET. When this is the case, it is possible to design a remarkably simple procedure for estimating this universal dependence. This procedure provides a practical method for analyzing experimental data without using the approximate equation (3). Specifically, the function $E_{\text{obs}}(\tau_D^{\text{intr}}/\tau_R, \sqrt{\langle R^2 \rangle}/R_0)$ can be estimated from a single, sufficiently long simulation trajectory $R(t)$ for *any* polymer from the same universality class as the system of interest. This is true because, instead of varying the properties of the polymer itself (e.g., the number of monomers, persistence length, or solvent friction), one can take advantage of the universality of the function and vary the ratios $\tau_D^{\text{intr}}/\tau_R$ and $\sqrt{\langle R^2 \rangle}/R_0$ by changing τ_D^{intr} and R_0 while using exactly the same polymer trajectory.

The rest of this paper is organized as follows. In Sec. II we describe the general theory for the FRET efficiency in moving polymers. In Sec. III we present our numerical results for unfolded polypeptides and show how dimensional analysis can be used to recast those results in a universal form. Finally, Sec. IV discusses implications of our findings for experimental studies employing FRET to measure distances within unfolded proteins.

II. THEORY

In the following discussion, we will assume that our system is ergodic; that is, that time averages carried out over single-molecule trajectories recapitulate ensemble averaged properties. Suppose the donor is excited to state D^* at $t=0$. It can either directly decay back to the ground state D or undergo FRET to the acceptor A

For a given polymer trajectory $R(t)$, the probability of still remaining in state D^* by time t is given by

$$S(t) = \exp \left[-\Gamma t - \int_0^t k_{\text{FRET}}[R(t')] dt' \right]. \quad (4)$$

The probability of exciting the acceptor between t and $t+dt$ is given by

$$\begin{aligned} p_{\text{FRET}}(t) dt &= k_{\text{FRET}}[R(t)] dt S(t) \\ &= k_{\text{FRET}}[R(t)] dt \exp \left[-\Gamma t - \int_0^t k_{\text{FRET}}[R(t')] dt' \right]. \end{aligned} \quad (5)$$

Neglecting nonradiative decay mechanisms and assuming the weak excitation limit, in which the acceptor is likely to emit a photon before the donor becomes excited again,¹⁶ the observed FRET efficiency is then obtained by integrating $p_{\text{FRET}}(t)$ and averaging over the equilibrium distribution $p_{\text{eq}}[R(0)]$ for the polymer's initial end-to-end distance and over the stochastic trajectories $R(t)$. We will denote this average by angular brackets,

$$\begin{aligned}
E_{\text{obs}} &= \left\langle \int_0^\infty p_{\text{FRET}}(t) dt \right\rangle \\
&= \left\langle \int_0^\infty dt k_{\text{FRET}}[R(t)] \exp \left\{ -\Gamma t - \int_0^t k_{\text{FRET}}[R(t')] dt' \right\} \right\rangle \\
&= 1 - \Gamma \int_0^\infty dt \exp(-\Gamma t) S_{\text{FRET}}(t),
\end{aligned} \quad (6)$$

where we have defined

$$S_{\text{FRET}}(t) = \left\langle \exp \left\{ - \int_0^t k_{\text{FRET}}[R(t')] dt' \right\} \right\rangle. \quad (7)$$

The latter expression is the survival probability of the donor assuming that FRET is the only decay process. Another useful form of Eq. (6) is

$$E_{\text{obs}} = - \int_0^\infty \frac{dS_{\text{FRET}}}{dt} \exp(-\Gamma t) dt. \quad (8)$$

Finally, yet another remarkably simple relationship^{2,4,23,25} exists for E_{obs}

$$E_{\text{obs}} = 1 - \Gamma \bar{\tau}_D = 1 - \frac{\bar{\tau}_D}{\tau_D^{\text{intr}}}, \quad (9)$$

where $\bar{\tau}_D$ is the mean donor lifetime in the presence of the acceptor (as defined below). Equation (9) remains exact regardless of the time scale on which the donor-acceptor distance fluctuates and irrespective of the functional character (e.g., exponential, multiexponential, and stretched exponential) of the lifetime decay curve. To prove this, notice that the probability that the donor will de-excite between t and $t+dt$ is given by $p(t)dt = -(dS/dt)dt$, where $S(t)$ is given by Eq. (4). Thus the mean donor lifetime is given by

$$\bar{\tau}_D = \int_0^\infty t p(t) dt = \int_0^\infty S(t) dt. \quad (10)$$

It is then easy to see that Eq. (9) is identical to Eq. (6).

Equations (6)–(10) provide complete formal solution for the observed FRET efficiency. Before proceeding to their numerical evaluation, let us consider the extreme cases of long and short donor lifetimes.

- (A) The limit of long donor lifetime, $\Gamma \rightarrow 0$. In this case, using Eq. (8) and assuming Γ to be an independent parameter we find $E_{\text{obs}} = -\int_0^\infty (dS_{\text{FRET}}/dt) dt = 1$. The measured FRET efficiency is 1 regardless of the actual donor-acceptor distance, as predicted above on the basis of more qualitative arguments.
- (B) The limit of short donor lifetime, $\Gamma \rightarrow \infty$. In this limit, many photons are emitted before the protein moves significantly. The integrand of Eq. (6) decays over a short time scale, $t \sim 1/\Gamma$, during which the protein remains essentially frozen. To estimate the survival probability in this case we replace the integral $\int_0^t k_{\text{FRET}}[R(t')] dt'$ in Eq. (7) by simply $k_{\text{FRET}}(R)t$

$$\begin{aligned}
S_{\text{FRET}}(t) &\approx \langle \exp(-k_{\text{FRET}} t) \rangle \\
&= \int_0^\infty dR p(R) \exp[-k_{\text{FRET}}(R)t].
\end{aligned} \quad (11)$$

Substituting this into Eq. (6) we find

$$E_{\text{obs}} = \int_0^\infty dR p(R) \frac{k_{\text{FRET}}(R)}{k_{\text{FRET}}(R) + \Gamma} = \left\langle \frac{k_{\text{FRET}}(R)}{k_{\text{FRET}}(R) + \Gamma} \right\rangle, \quad (12)$$

which is identical to the standard approximation of Eq. (3).

III. RESULTS

We are not aware of any simple analytical approximations for Eq. (6) in the general case. Indeed, even the approximate solution represented by Eq. (3) cannot be generally evaluated analytically except in certain asymptotic limits.^{5,13} To examine how the observed FRET efficiency depends on the donor lifetime and on the polymer dynamics we thus instead resort to simulations. We have used Langevin dynamics to simulate FRET kinetics for donor-acceptor pairs located at the ends of polypeptide chains in water. The model that we used is similar to those in Refs. 26 and 27. Those models treat each amino acid residue as a single hydrophobic, hydrophilic, or neutral bead and approximately capture the dihedral preferences of polypeptide chains as well as excluded-volume effects. As we are interested in unfolded, noninteracting polypeptides, we employed only neutral beads. The friction coefficient per each bead was set to that of water as previously estimated in Refs. 27 and 28.

Since excluded-volume interactions are present in the model, sufficiently long polypeptides obey the Flory statistics.²⁹ Thus, the root-mean-square end-to-end distance obeys the relationship

$$\langle R^2 \rangle^{1/2} = a(N-1)^\nu, \quad (13)$$

where N is the number of monomers and $\nu=0.588$. In the limit where the intrinsic donor lifetime is short compared to the time scale of polymer reconfiguration, the FRET efficiency is given by the average over the equilibrium probability distribution [Eq. (3)]. In this case, the value of $E_{\text{obs}} = \langle E \rangle$ depends only on the dimensionless ratio of the characteristic polymer size, $\langle R^2 \rangle^{1/2}$, and the length scale of FRET given by the Förster radius R_0 , as long as both of these characteristic lengths are much longer than the monomer size. Indeed, when the length of the polymer was increased from $N=20$ to $N=40$ and the Förster radius was simultaneously rescaled from $R_0=20$ Å to $R_0=20$ Å $\times (39/19)^{0.588} \approx 30.5$ Å to preserve the dimensionless ratio $\rho = \langle R^2 \rangle^{1/2}/R_0$, this resulted in the same value of E_{obs} (Fig. 1) in the limit of very short donor lifetimes. This value also coincides with the average $\langle E \rangle$ numerically computed from Eq. (3).

As the donor lifetime is increased (while keeping the value of the Förster radius fixed), the observed FRET efficiency deviates from the static limit of Eq. (3). We note that this is numerically equivalent to the more difficult, if more

FIG. 1. The observed FRET efficiency increases with the intrinsic lifetime of the donor, τ_D^{intr} , which is the lifetime of the donor in the absence of the acceptor. As shown, this effect is more pronounced for shorter polymer chains ($N=20$) than for longer chains ($N=40$). Note that the Förster radius employed here was chosen such that in the static limit ($\tau_D^{\text{intr}} \ll \tau_R$), the limit in which Eq. (3) is an accurate approximation, the FRET efficiency of both cases is the same.

relevant, problem of maintaining a constant intrinsic donor lifetime while altering the time frame of polymer dynamics (e.g., as the solvent viscosity is changed via the introduction of urea^{2,7,9,11}). For shorter chains, such as $N=20$, we find deviations of order of 30% for the 1–3 ns donor lifetimes of typical FRET experiments. This effect becomes less significant for longer chains; it falls to 15% over this same time scale, for example, for a 40-residue chain.

To understand how chain length affects the dependence of E_{obs} on the donor lifetime we note that the characteristic time of polymer dynamics τ_R is itself strongly dependent on chain length. Within the Rouse model a polymer exhibits a spectrum of relaxation times with the slowest given by^{29–31}

$$\tau_R = \frac{1}{3\pi^2} \frac{\langle R^2 \rangle}{D}, \quad (14)$$

where D is the diffusion coefficient for the chain as a whole, which is inversely proportional to the number of monomers, N . Because the FRET efficiency E_{obs} is a dimensionless quantity, we expect it to be dependent only on the ratio $T = \tau_D^{\text{intr}}/\tau_R$. Since for longer chains the time τ_R is also longer, the error $E_{\text{obs}} - \langle E \rangle$ introduced by the approximation of Eq. (3) should be smaller for the same value of τ_D^{intr} . Based on the above argument, we also expect that if the dependence shown in Fig. 1 is rescaled and plotted as a function of $\tau_D^{\text{intr}}/\tau_R$, the resulting curve should be the same for both values of chain length. This is indeed the case (Fig. 2), except for rather small discrepancies between the two curves pre-

FIG. 2. Polymers differing in length ($N=20$ and $N=40$) exhibit (nearly) the same dependence on chain dynamics when the observed FRET efficiency is plotted as a function the dimensionless ratio $\tau_D^{\text{intr}}/\tau_R$ of donor lifetime and the polymer reconfiguration time (data as in Fig. 1). The solid line is a guide to the eye.

FIG. 3. The FRET efficiency calculated from Eqs. (6)–(9) (circles) deviates from the prediction of the commonly employed Eq. (3) (squares) even when the donor lifetime is considerably shorter than time scale associated with polymer fluctuations (here $T = \tau_D^{\text{intr}}/\tau_R = 0.2$ and $N=40$). Of note is that these curves are virtually independent of the chain length). Here, horizontal lines correspond to $E_{\text{obs}}=0.1$ and $E_{\text{obs}}=0.6$.

sumably caused by finite-size effects and by Eq. (14) being only an approximation in the case of excluded-volume polymers.

Likewise, we expect that the characteristic length scales of FRET (R_0) and of the polymer chain ($\langle R^2 \rangle^{1/2}$) enter into the dimensionless function E_{obs} only via their dimensionless combination, $\rho = \langle R^2 \rangle^{1/2}/R_0$, as long as both of these lengths are much greater than the monomer size.²⁹ We thus conclude that $E_{\text{obs}}(T, \rho)$ expressed as a function of two dimensionless arguments should be a universal function that is independent of the precise nature of the polymer in question. This conclusion is of practical importance: This function, if obtained from *any* polymer model from the same universality class (e.g., Rouse chains), no matter how simplistic and unrealistic the model is, can be applied to the analysis of experimental data without loss of accuracy.

This universality can be exploited even further. All one needs to evaluate $E_{\text{obs}}(\tau_D^{\text{intr}}/\tau_R, \sqrt{\langle R^2 \rangle}/R_0)$ is a single, sufficiently long trajectory $R(t)$ for a polymer of any length. Indeed, given a trajectory $R(t)$ the evaluation of $\sqrt{\langle R^2 \rangle}$ is straightforward. Moreover, the time τ_R can be estimated directly from $R(t)$ by following the time dependence of the autocorrelation function of the end-to-end distance.³² The FRET efficiency $E_{\text{obs}}(T, \rho)$ can then be estimated for any value of T and ρ from Eqs. (6)–(9) by varying the parameters τ_D^{intr} and R_0 of the FRET pair while keeping the properties of the polymer fixed. Critically, the dependence $E_{\text{obs}}(T, \rho)$ thus obtained will be directly transferrable to other polymers.

To illustrate this approach, we have computed the dependence of $E_{\text{obs}}(T, \rho)$ on ρ for $T = \tau_D^{\text{intr}}/\tau_R = 0.2$ (Fig. 3). For a fixed value of T , one can use the relationship between $E_{\text{obs}}(T, \rho)$ and ρ to estimate the root-mean-square distance $\langle R^2 \rangle^{1/2}$ for the unfolded polypeptide from the measured value of E_{obs} , as illustrated in Fig. 3 for $E_{\text{obs}}=0.1$ and $E_{\text{obs}}=0.6$. We find that even when, as assumed here, τ_R is five times the intrinsic donor lifetime, the errors in estimating $\langle R^2 \rangle^{1/2}$ introduced by using the approximation of Eq. (3) can be $\sim 10\%–25\%$,

IV. DISCUSSION

While single-molecule FRET measurements offer a powerful experimental technique for measuring distances within unfolded proteins and other biopolymers,² their interpretation

heavily relies on theoretical models of the unfolded state and of the FRET kinetics, whose validity has not always been directly established. Understanding to what extent limitations of those models limit the accuracy of the FRET-derived distances is especially important in view of the significant disagreement between small angle x-ray scattering experiments, which suggest that the dimensions of chemically denatured proteins, such as protein L, are effectively independent of the denaturant concentration³³ and the results of a number of single-molecule FRET experiments that have been interpreted as suggesting that the denatured state of this and other proteins expands significantly as the concentration of the denaturant is increased.^{4,7,9,11,12}

Here, we have focused on the errors caused by the assumption that the polymer dynamics can be neglected on the time scale of donor emission so that the polymer is effectively static. This assumption has been previously made in many studies of unfolded proteins and polypeptides.^{2,4,7,8,13,21,34} To assess its validity it is necessary to evaluate the ratio $T = \tau_D^{\text{intr}} / \tau_R$ of the donor lifetime (in the absence of acceptor) and the polymer reconfiguration time. It is useful to rewrite T in terms of other quantities commonly reported in experimental studies. In particular, experiments probing end-to-end dynamics of polymers are commonly interpreted in terms of one-dimensional diffusion occurring in the potential of mean force, which is related to the distance distribution $p(R)$ via the formula $G(R) = -k_B T \ln p(R)$. Given an effective end-to-end diffusion coefficient $D_{\text{end to end}}$, the polymer reconfiguration time can be estimated using a harmonic oscillator approximation, $\tau_R = k_B T G'' / D_{\text{end to end}}$, where the second derivative of $G(R)$ should be evaluated at its minimum. For chains that obey Gaussian statistics, this gives⁷ $\tau_R = \langle R^2 \rangle / 6D_{\text{end to end}}$ so that

$$T = \tau_D^{\text{intr}} / \tau_R = \frac{6D_{\text{end to end}} \tau_D^{\text{intr}}}{\langle R^2 \rangle}. \quad (15)$$

The parameter of Eq. (15) (aside from the factor of 6) has been empirically introduced by Stryer *et al.*³⁵ (see also Ref. 36) to estimate the enhancement of FRET provided by diffusion. The physical meaning of Eq. (15) is simple: It is the ratio of the mean-square change in the donor-acceptor distance during the excited donor lifetime (which provides an estimate of the measurement error) and the mean-square donor-acceptor distance.

We now proceed with estimating the ratio $\tau_D^{\text{intr}} / \tau_R$ in order to assess the importance of polymer dynamics in several recent FRET studies. For dye molecules used in FRET experiments, typical values of τ_D^{intr} are in the range 1–5 ns. The reconfiguration time τ_R can be estimated experimentally using fluorescence correlation spectroscopy.^{7,8,16,17} For example, depending on the denaturant concentration the viscosity corrected values of τ_R reported in Ref. 7 are in the range 20–60 ns for the chemically denatured, single-domain protein CspB. Assuming that the uncorrected values are of the same order of magnitude, there is roughly an order of magnitude disparity between these two time scales and thus Eq. (3) appears to be a reasonably accurate approximation. Likewise, Merchant *et al.*⁴ estimated $\tau_D^{\text{intr}} / \tau_R = 3.78$ ns/50 ns = 0.076 for the unfolded state of protein L, another single-

domain protein. They further verified the validity of Eq. (3) by computing fluorescence decay kinetics using a simple one-dimensional diffusion model for the end-to-end distance dynamics and comparing the result with that obtained in the absence of any chain dynamics.

Although the above discussion suggests that, for single-domain proteins, τ_R is typically an order of magnitude greater than τ_D^{intr} and so the errors of the static approximation should be relatively small, we emphasize that Eq. (3) should still be used with caution. Indeed, in the example shown in Fig. 3 the ratio $\tau_D^{\text{intr}} / \tau_R = 0.2$ is consistent with the typical experimental ranges of τ_R and τ_D^{intr} yet using Eq. (3) leads to an error of 10%–25% in estimating the mean value of the end-to-end distance. Moreover, τ_R is strongly dependent on chain length. For example, within the Rouse model it is proportional to the square of the number of monomers N .^{29,31} Therefore we expect that violation of Eq. (3) would be common in FRET studies of short polypeptides. For example, Soranno *et al.*³⁷ reported on measurements of the kinetics of end-to-end collisions in polypeptides with $N = 14$. They interpreted their data in terms of an effective one-dimensional diffusion model and estimated the relative end-to-end diffusion coefficient to be in the range $D_{\text{end to end}} = 10\text{--}20 \text{ \AA}^2/\text{ns}$. Using the typical value³⁷ $\sqrt{\langle R^2 \rangle} = 20 \text{ \AA}$, the polymer reconfiguration time can be estimated as $\tau_R = \langle R^2 \rangle / 6D_{\text{end to end}} = 3\text{--}6$ ns. Therefore the value of τ_R for these short peptides is comparable to lifetimes of dyes often employed in FRET and thus the effect of polymer dynamics on the observed FRET efficiency may be important. In another recent study,³⁶ the value of $\tau_D^{\text{intr}} / \tau_R$ was estimated to be ≈ 0.42 in water (but more than an order of magnitude lower for propylene glycol) for a short peptide containing $N = 8$ residues, thus suggesting that dynamic effects may be significant. [See supporting information for Ref. 36, where the ratio $D_{\text{end to end}} \tau_D^{\text{intr}} / \langle R^2 \rangle$ is estimated. According to Eq. (15), this should be multiplied by a factor of 6 for a Gaussian chain.]

The above estimates made for short polypeptides should, however, be taken with a grain of salt because the dependence of E_{obs} on $\tau_D^{\text{intr}} / \tau_R$ is expected to be universal only in the $N \rightarrow \infty$ limit. In addition, those polymers are unlikely to be accurately described by Gaussian statistics and so Eq. (15) may not apply. Finally, the length of the linkers connecting the peptides to the FRET dyes, as compared to the peptide length, becomes non-negligible, thereby further complicating the situation.

In summary, we have shown here that neglecting polymer dynamics can cause significant errors in FRET-derived distances within short unfolded biopolymers and described the means by which this problem can be remedied. The magnitude of these errors depends on both the time scale of polymer dynamics relative to that of donor emission and on the FRET length scale relative to the mean distance that is being measured. We hope that the analysis presented here will help improve the accuracy of single-molecule FRET measurements.

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