A review of the analysis of complex time-resolved fluorescence anisotropy data

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1. Introduction

The absorption of polarised electromagnetic radiation by an ensemble of randomly oriented fluorescent molecules will result in the preferential excitation of those molecules that have a vector component of their absorption transition moment oriented parallel to the plane of the electric field of the incident excitation light. 

A given fluorophore will also have an emission transition moment, which may or may not be aligned parallel to that of the absorption transition in the molecular structure frame of reference. Following this ‘photoselection’ of molecules from the ensemble, some degree of emission polarisation relative to the orientation of the initially photoselected chromophores will be exhibited. Assuming vertically polarised photoexcitation, in the usual ‘L’ or ‘T’ configurations used for the majority of polarised emission measurements in a conventional fluorimeter, the degree of polarisation of the emission, P, is defined as:

\[ P = \frac{I_{\parallel} - G I_{\perp}}{I_{\parallel} + G I_{\perp}} \]  

(1)

where \( I_{\parallel} \) and \( I_{\perp} \) are the intensities of the emission with the polarisation analyser oriented parallel and perpendicular to the plane of the excitation electric field respectively and \( G \) is the correction factor included to account for any intensity bias of the detection system (in particular, lenses or a monochromator grating) towards one polarisation over the other.

Following photoselection, the emission from a fluorophore embedded in a rigid environment such as a polymer matrix or low temperature glass, exhibits an inherent degree of polarisation of its emission. This ‘limiting anisotropy’, \( r_0 \), of the fluorophore reflects the angle between the absorption and emission transition moments within the coordinates defined by the molecular structure. From equation (2), assuming \( G = 1 \), it can be seen that under the extreme cases, the value of \( r \) can vary between 1 and \(-1/2\), or zero for complete depolarisation. The two extreme values can only be observed from a molecularly aligned system.
which the angle between the absorption and emission transition moments is zero or 90°, respectively, and are therefore not observed in conventional, molecularly randomised circumstances. The distribution of the angles of the transition moments associated with photooselection and emission means that the observable values of \( r_0 \) can range from 0.4 for parallel absorption and emission transition moments to \(-0.2 \) when these transitions are at right angles to one another. The value of \( r_0 \) for a given fluorophore can be determined from the intercept of a Perrin plot (following the anisotropy being recorded as a function of temperature or viscosity) [2] or from highly aligned samples [3].

Time-resolved fluorescence anisotropy measurements can provide a direct measure of the dynamics of processes that result in the time-dependent loss of polarisation of the molecules’ emission following photooselection by polarised excitation. Measurements of the time-dependence of emission depolarisation can be performed in either the time- or frequency- domains. In this paper we restrict ourselves to discussion relating to the time-domain, but similar approaches can be invoked for the frequency domain [2]. In terms of time domain measurements, the fluorescence anisotropy is expressed as:

\[
I(t) = \frac{r(t) - 2r_0}{1 - 2r_0} I(0)
\]

When the fluorophore is embedded in a rigid environment (low temperature glass or polymer matrix) and at low concentrations, determining the fluorescence anisotropy as a function of time after photoexcitation should result in a time-invariant (constant) value corresponding to \( r_0 \). A number of phenomena can lead to the time-dependent depolarisation of the emission, leading to \( r(t) \) trending to zero from the initial value at \( t = 0 \) of \( r_0 \), characteristic of the fluorophore. Such depolarising processes include rotational motion of the fluorophore, or energy migration/hopping between like fluorophores (homo-FRET), occurring on the timescale of the lifetime of the excited state. In the presence of rotational motion or energy migration, monitoring the change in \( r \) as a function of time gives a direct measure of the time-scale of these depolarising processes. For time-resolved fluorescence anisotropy measurements the time-scale of the emission depolarising process should therefore be of a similar order of magnitude to the fluorescence decay time of the fluorophore probing that process. If the emission lifetime is significantly shorter than the time-scale of the depolarising process, then there is no emission to report at long times and if the emission lifetime is considerably longer than the emission depolarising process dynamics the changes in \( r(t) \) will happen before the excited state can report truly on the dynamics.

The intensities of the parallel (||) and perpendicularly (⊥) polarised components of the emission as a function of time following ultrashort pulsed photoexcitation are given by equations (4) and (5), respectively.

\[
I(t) = \frac{1}{3} I(0)[1 + 2r(t)]
\]

\[
I_\perp(t) = \frac{1}{3} I(0)[1 - r(t)]
\]

where \( I(t) \) is the total fluorescence intensity decay function given by \( I(t) = I_0(t) + 2I_\perp(t) \) and \( r(t) \) is the time-resolved anisotropy function. The \( I(t) \) function can often show complex (non-exponential) decay behaviour and the \( r(t) \) profile, calculated from equation (3), is generally expected to decay in time towards zero, often modelled by a sum of exponential terms. The combination of complex functional forms for \( I(t) \) and/or \( r(t) \) leads to quite complicated decay functions for both \( I_0(t) \) and \( I_\perp(t) \) for even reasonably ‘simple’ systems. The form of \( r(t) \) can therefore be a more direct visualisation of the dynamics of the depolarisation process.

In this review we show several examples where straightforward fluorescence anisotropy behaviour is not observed in the time domain, even from systems that superficially look remarkably simple. We concentrate on three phenomena that can give rise to a complex time-dependence of the depolarisation of the emission of simple fluorophores, namely molecular rotational motion, excitation energy transfer and inherent photophysics of the fluorophore. This paper reviews observations from within our own laboratories of unexpectedly complex fluorescence anisotropy decay behaviour from relatively simple systems and describe the mechanisms that give rise to these results.

2. Molecular rotational motion

Rotational motion of a fluorophore is the most commonly studied phenomenon that leads to a time-dependent depolarisation of fluorescence. Following photooselection, if the fluorophore undergoes rotational motion on the time-scale of its excited state lifetime, its emission anisotropy, \( r(t) \), will decay in time from its initial value of \( r_0 = r(0) \) to zero (corresponding to complete depolarisation of the emission) [4]. When the sample exhibits a single rotational mode (assuming a spherical molecular structure) the \( r(t) \) profile decays exponentially (equation (6)) with a decay constant corresponding to the rate that the photooselected molecules re-randomise the orientation of their emission transition moments. The rotational correlation time, \( \tau_{\text{rot}} \), is the time taken for the initial anisotropy to fall to 1/e of its original value due to molecular rotation. The alternative expression substitutes the Debye rotational relaxation time, \( \rho \), for the rotational correlation time (\( \rho = 3\tau_{\text{rot}} \)) which is the time for a given orientation to rotate through an angle given by the arc cos \( e^{-1}(68.42°) \) [5].

\[
r(t) = r_0 \exp\left(-\frac{t}{\tau_{\text{rot}}}\right)
\]

Under this condition and assuming a single fluorescence decay component for \( I(t) \) (single fluorescence lifetime,
\( \tau_i \), the fluorescence decay profiles of \( I_T(t) \) and \( I_T(t) \) (equations 4 and 5) are then both double exponential decay functions (sums and differences of exponential components, respectively). However, it is clear that the functional forms for both the fluorescence decay profiles, \( I_T(t) \) and \( I_T(t) \), and \( r(t) \) quickly become quite complex for even slightly more complicated systems.

The fluorescence decay behaviour of some fluorophores can be sensitively affected by properties of the local environment they experience, including pH, viscosity, ion concentration, dielectric constant and others. If the fluorophore experiences more than one local environment its fluorescence decay behaviour can be non-exponential and the expressions for the polarised fluorescence decay profiles, \( I_T(t) \) and \( I_T(t) \), equations (4) and (5), become very complex. This is exacerbated if the different environments also lead to different rotational behaviour of the fluorophore, or the shape of the rotating molecular system being probed by the fluorophore is not spherical. The \( r(t) \) profile is generally described in terms of a multi-exponential decay function to account for these different modes of fluorophore rotation (or asymmetric or ellipsoidal rotors) \[4\]:

\[
\begin{align*}
  r(t) &= r_0 \sum \frac{g_j}{r_0} \exp \left( -\frac{t}{\tau_j} \right) = \sum \frac{r_0}{r_0} \exp \left( -\frac{t}{\tau_j} \right) \\
  \text{where } r_0 &= \sum r_0 \text{ is the limiting anisotropy in the absence of rotational diffusion, } \tau_{rot} \text{ are the individual rotational correlation times the fluorophore experiences and } g_j \text{ are the fractional amplitudes of each rotational correlation time in the anisotropy decay ( } \sum g_j = 1.0 \text{).}
\end{align*}
\]

Whilst there are examples in the literature illustrating the analysis of fluorescence anisotropy decays of systems exhibiting multiple fluorescence lifetimes and rotational correlation times, the \( r(t) \) profiles still usually decay towards zero, indicating eventual complete depolarisation of the emission. We show here two relatively simple examples of molecular rotation where the \( r(t) \) profiles do not just decay to zero as expected.

The adsorption of a fluorescent dye from solution to a solid substrate can influence the fluorescence lifetime of the dye considerably. The radiative rate constant, \( k_{rad} \), may, or may not be influenced by the presence of the solid/liquid interface, but it is highly likely that the non-radiative rate constant, \( k_{non} \), can be altered significantly due to the adsorption. One classic example is the adsorption of Malachite Green to a silica surface \[6\]: in bulk solution this dye has a very low fluorescence quantum yield and a concomitantly short fluorescence lifetime. Upon adsorption the quantum yield and fluorescence lifetime become remarkably larger. This can be explained in terms of the reduction in the vibrationally induced energy relaxation of the excited state of the Malachite Green due to the structural interactions resulting from attachment of the dye to the interface. The fluorescence lifetime of a dye near an interface can also be affected by the influence of refractive index on the radiative rate constant described through the Strickler–Berg relationship (equation (8)) \[7\].

\[
k_{rad} = 2.88 \times 10^{-9} n^2 \int \frac{F_i(\nu_f) d\nu}{\nu^3 F_i(\nu_f) d\nu} \int \frac{\epsilon(\nu_f) d\nu}{k_A} (8)
\]

where \( n \) is the refractive index, \( F_i \) is the fluorescence intensity per unit wavenumber, \( \nu, \epsilon(\nu_f) \) is the extinction spectrum. In the context of the adsorption of a dye to a solid interface, the dye may experience a different refractive index at (or refractive index gradient with distance from) the interface, thus influencing \( k_{rad} \) and thereby, the fluorescence lifetime, \( \tau_f \). The fluorescence lifetime of some dyes can also be highly sensitive to local pH or polarity effects, which might be expected in the vicinity of an interface, or energy and/or electron transfer between the fluorescent dye (e.g. tetramethyl rhodamine) and the substrate \[8, 9\]. Effects such as homo-energy transfer, enhanced due to a high local fluorophore concentration caused by adsorption, could also affect the fluorescence decay time behaviour of an adsorbed probe. The radiative rate constant can also be influenced by the presence of regions of variable dielectric permittivity, such as an interface. The electromagnetic field surrounding an excited oscillating dipole can become distorted due to the imposed interfacial boundary condition \[10, 11\]. This principally results in the electromagnetic field being reflected, which subsequently interacts with the dipole to either enhance or inhibit the viability of the excited state \[12–14\]. The presence of the interface will therefore influence possible relaxation processes, which in turn alter the quantum efficiency of the fluorophore. If the electromagnetic field reflection returns in phase with the oscillating dipole, the dipole is driven in part by this reflection and hence the emission will be enhanced leading to an increase in \( k_{rad} \) \[10, 15–18\]. In general, a dielectric interface can result in a complex (dampened, sinusoidal-like) dependence of the \( k_{rad} \) and thereby the \( \tau_f \) on distance from the interface \[11\].

### 2.1. Adsorption of a dye to nanoparticles

The application of time-resolved fluorescence anisotropy measurements (TRAMs) to the investigation of the adsorption of the dye Rhodamine B (RhB) and a RhB-labelled cationic polyelectrolyte onto colloidal silica (Ludox) has been described \[19\]. This represents a dramatic example of the complex time-dependence of \( r(t) \) from a quite ‘simple’ system. Fluorescence anisotropy decay curves, \( r(t) \), of dilute RhB in water in the absence and presence of varying amounts of added Ludox HS-30 are presented in figure 1.

In the absence of Ludox, the fluorescence anisotropy signal decays rapidly to zero, as expected for a simple (spherical) emitting rotor in solution. A single exponential fit of this decay gives a rotational correlation time of \(~300\) ps, which is typical of the fast
depolarising rotation of free RhB in aqueous solution. In the presence of Ludox, however, the anisotropy does not decay completely to zero, but instead regains intensity with time. This behaviour can be explained as follows. The RhB can be considered as experiencing two distinct environments; free in solution, but also adsorbed to the colloidal silica particles. The dye exhibits a significantly different fluorescence decay time when it is adsorbed to the silica, compared to bulk solution, for one or more of the reasons discussed above. In addition to the two fluorescence decay times corresponding to the two environments for the fluorophore, it will also undergo two significantly different modes of rotational motion; rotation of the free fluorophore in solution and when the fluorophore is adsorbed to the colloidal silica particle. The latter mode of rotation would be expected to be somewhat slower than the former, as the fluorophore is reporting on the overall motion of the silica particle to which it is adsorbed. For a heterogeneous system, $r(t)$, is a linear combination of the anisotropy decays of each lifetime species in the mixture:

$$r(t) = \sum_{i=1}^{n} f_i(t) \tau_i$$

(9)

where $f_i(t)$ is the ratio of the decay law for species $i$ to the decay law for the entire system [20]:

$$f_i(t) = \frac{a_i \tau_i(t)}{\sum_{j=1}^{n} a_j \exp(\tau_j)} = \frac{a_i \tau_i(t) + 2 I_a(t))}{\sum_{j=1}^{n} a_j \exp(\tau_j)}$$

(10)

and $\sum_{i=1}^{n} f_i(t) = 1$. In the current case of RhB/silica particles, $n = 2$ and we refer to the two fractions as the ‘free’ and ‘adsorbed’ forms, $f_f(t)$ and $f_a(t)$, respectively.

An analytical form for $r(t)$ can then be derived:

$$r(t) = f_f(t) \tau_f(t) + f_a(t) \tau_a(t)$$

(11)

where $f_f(t)$ is the fraction of fluorescence at time $t$ due to free RhB, $r_f(t)$ describes the anisotropy decay of these free probes and $f_a(t)$ and $\tau_a(t)$ are the corresponding quantities [21], in this case for RhB bound to the colloidal particles. The fraction term for the free dye is given by equation (12) and an analogous expression describes the fraction term for the adsorbed form:

$$f_i(t) = \frac{x_f \sum_{j=1}^{n} a_j \exp(\tau_j)}{x_f \sum_{j=1}^{n} a_j \exp(\tau_j) + x_a \sum_{j=1}^{n} a_j \exp(\tau_j)}$$

(12)

where $\tau_f$, $\tau_a$ and $\tau_{\text{rot}}$, $\alpha_{\text{rot}}$ are the corresponding fluorescence lifetimes and amplitudes for the two (free and adsorbed) populations of probes. Immediately after excitation ($t = 0$), $f_f(t)$ and $f_a(t)$ are the actual fractions of the free and adsorbed probe molecules, $x_f$ and $x_a$, respectively, assuming that the extinction coefficient and radiative rate constant are the same for both populations. In the present case, we are dealing with a single rotational correlation time and a single fluorescence decay time for each of the two forms of the probe, so $N = 1$ in equation (12).

The solid lines in figure 1 are the fits to the various $r(t)$ profiles using a ‘global analysis’ approach, comprising two common $\tau_f$ values and two common $\tau_{\text{rot}}$ values. The differences between the curves represent the variation in the fractions, $f_f(t)$, of the two terms (equation (12)). The ‘dip-and rise’ behaviour comes about because, with time after photoexcitation, the fluorescence decay of the shorter lived species diminishes relative to that of the longer-lived species to the point that at later times there is little to no emission emanating from the short-lived species to report on its motion. The $r(t)$ intensity therefore increases with time reflecting the increasing dominance of the contribution of the longer-lived (and more slowly...
rotating) species to the overall value of the fluorescence anisotropy. Such ‘dip-and-rise’ behaviour in fluorescence anisotropy profiles has been reported previously, particularly in the biophysical literature. Hudson et al observed some evidence of this behaviour in studies of lipid bilayers [22, 23] and in the same year Brand et al [24] showed that the dynamics of perylene and 9-aminoacridine in glycerol exhibited similar ‘dip and rise’ behaviour based on rotational modes of motion. The basis behind the ‘anomalous’ anisotropy decays was identified soon after [21] and a series of subsequent studies have built upon these pioneering papers, interpreting quite pronounced complex time-resolved fluorescence anisotropy data based on this ‘associative’ fluorophore rotational model. Hudson and co-workers continued their work investigating the dynamics of trans-parinaric acid in lipid bilayers and showed marked ‘dip-and-rise’ behaviour that was readily interpreted in terms of this associative model [25].

Millar et al have investigated the interaction of DNA with the Klenow fragment of DNA polymerase I using the emission from dansyl-labelled DNA templates [20, 26, 27], from which they have observed marked ‘dip and rise’ behaviour, again interpreted on the basis of the associated model. Visser et al have made extensive use of TRAMs to investigate micelles and rat skeletal muscle membranes [28, 29], octadecyl rhodamine B in the bacteriophage M13 coat protein reconstituted in phospholipid bilayers [30, 31]. Krisnamoorthy et al [32] have evaluated the cytoplasmic viscosity of cells using TRAMs of Kiton Red at a microscopic level and attributed the observed ‘dip-and-rise’ behaviour to the dye being ‘free’ and bound to macromolecules in the cell. This group has also studied the interpoly peptide interactions in amyloid protofibrils [33] using TRAMs to probe the rotational dynamics of the environment-sensitive fluorophore 1,5-IAEDANS attached to specific locations in the protein, as a function of protein concentration. Quite dramatic changes in the $r(t)$ profile, tending to marked ‘dip-and-rise’ behaviour, was observed as a function of time as the protein aggregated and related to the rotational freedom of the fluorescent probe. Other examples in which complex $r(t)$ profiles have been observed and interpreted in terms of this rotational picture include studies of the microenvironment of cytochrome c oxidase and cardiolipin [34], the conformational dynamics of a flexible reactive centre loop of plasminogen activator inhibitor-1 [35], the interaction of bovine serum albumin with dipolar molecules [36], and most recently from ethidium bromide interacting with DNA [37].

Similar fluorescence anisotropy profiles have also been reported from other scenarios including excited state intramolecular proton transfer emission as a molecular reporter for probing of microheterogeneous environments of lipid-bilayer systems [38] and studies of electrostatically and covalently labelled silica nanoparticles [39].

### 2.2. Solubilised fluorophore in a polymer host

We have also observed similar ‘dip-and-rise’ behaviour in the time-resolved fluorescence anisotropy data recorded from pyrene (as a model, hydrophobic guest) solubilised by the polymer poly(N-isopropylacrylamide), PNIPAM [40]. PNIPAM, exhibits thermo-reversible phase separation in aqueous solution [41] characterised by a low critical solution temperature (LCST) of ~31–32 °C. Individual collapsed PNIPAM coils exist in ultra-dilute aqueous solutions above the LCST of the polymer and below the LCST the polymer structure expands. The PNIPAM therefore has the capacity to solubilise organic guest species in its collapsed state (above its LCST) and undergo controlled release of these species triggered by a temperature change to below the LCST. Fluorescence decay measurements of pyrene, dispersed at $10^{-6}$ M in $10^{-3}$ wt% solutions of PNIPAM, indicated that the pyrene fluorescence reports on at least two different environments. ‘Force-fitting’ of the fluorescence decay measured at 37 °C (i.e. above its LCST) to a dual exponential decay function afforded fluorescence lifetimes of ~82 and 218 ns for pyrene within this dilute PNIPAM system. The former value was loosely associated with pyrene molecules located within the bulk aqueous phase. The longer-lived species were deemed to be representative of excited states of the solute/probe solubilised within the intramolecular hydrophobic domains created within the PNIPAM above its LCST. Dual exponential fitting of the fluorescence decay from the dispersed pyrene at 24 °C (i.e. below its LCST) resulted in estimates of ~28 and 135 ns, respectively, for the excited state lifetimes characterising the distribution of the pyrene solute species in the aqueous dispersion. The latter value is close to that of ~130 ns obtained as the ‘average fluorescence lifetime’ of pyrene ($10^{-6}$ M) in water, at this temperature. The former lifetime of ~30 ns is frequently recovered in multi-exponential analyses of aqueous dispersions containing pyrene at high dilutions. The description that emerges for this system is therefore reminiscent of the previous section: the fluorescent probe experiences (at least) two environments of different fluorescence lifetimes and significantly different degrees of rotational restriction. Similarly complex time-resolved TRAMs signal to that of the previous example may therefore be exhibited and this behaviour might be expected to vary with temperature.

TRAMs measurements on these pyrene/PNIPAM solutions were performed above and below the LCST, with the resulting fluorescence anisotropy profiles shown in figure 2. In each case the $r(t)$ profile attains a minimum but finite value, some 40 ns after excitation of the pyrene. Thereafter, over a timescale of hundreds of nanoseconds, the fluorescence anisotropy increases with time. The complexity of the time dependence of anisotropy of fluorescence from pyrene, above the LCST of the system, reflects the heterogeneous nature...
of the medium in which the probe is dispersed. At one extreme, pyrene molecules are located within the bulk aqueous phase, with a short fluorescence decay time and rapid rotational mobility. At the other extreme, pyrene molecules are occluded within the hydrophobic interiors of the collapsed PNIPAM globules, exhibiting longer fluorescence decay times and slower rotational mobility. At short times of analysis, the time-dependence of the fluorescence anisotropy is dominated by the shorter-lived and faster rotating, pyrene probes, located within the bulk aqueous phase and/or more polar regions of the water-permeated coils of the PNIPAM. The initial rapid loss in emission anisotropy therefore reflects the significant contribution from these shorter-lived and rapidly tumbling solutes dispersed largely in the aqueous phase. At longer times, fluorescence from pyrene species solubilised within the highly viscous and ‘protective’ hydrophobic domains, created within the ‘collapsed’ PNIPAM host, would dominate the observed anisotropy. The subsequent enhancement in the $r(t)$ at longer sampling times, (figure 2) results from the ever-increasing dominance of fluorescence from these longer-lived, slowly rotating probes solubilised within the highly viscous cores of the globular conformation of the macromolecule. This behaviour can be modelled analytically based on the approach represented by equation (12) assuming a two-site model for above the LCST (solid line of best fit for data in blue in figure 2).

Below the LCST, the TRAMs data are even more complex than above it. At 297 K, most of the pyrene guest molecules are released to the bulk aqueous phase but some proportion of the organic guests remains associated with the (otherwise) open coils of the polymer. An adequate description of the $r(t)$ profile at this temperature could be achieved only when a minimum of a three-state model was adopted, which produced $\tau_c$ values of <1 ns, 220 ns and >20 ms. These serve not only to parameterise the anisotropy decay but also demonstrate the broad range of environments inhabited by the excited state probes and show that a small, but finite, proportion of the overall pyrene population, reorients very slowly. These pyrene molecules are closely associated with the polymer, presumably through hydrophobic interaction and their motion seems to reflect whole molecule tumbling of the PNIPAM in solution.

These anisotropy data confirm that, above the lower critical solution temperature (LCST) of PNIPAM, the polymer host (in the form of single, ‘collapsed’ macromolecules) is capable of solubilising hydrophobic guests, which can be at least partially released upon the induction of a conformational change in the polymer’s structure by a lowering of the temperature.

As discussed above, this ‘dip-and-rise’ behaviour in TRAMs signals has been reported elsewhere from a number of scenarios, most commonly involving molecular rotational motion. Szabo [42] has developed a comprehensive formalism to describe the temporal profile of the fluorescence anisotropy in macroscopically isotropic systems where both excited state and orientational dynamics contribute to the depolarisation. This formalism included energy transfer, heterogeneity and interconversion of excited states with different emission characteristics as well as both overall and internal reorientation. In the following sections we discuss a number of cases where similarly complex TRAMs behaviour is exhibited, in which molecular motion is not the primary cause.

3. Excitation energy transfer/migration

A polymer molecule, be it a synthetic aromatic polymer or a biomacromolecule, consisting of repeating units of chromophores, represents a very high local concentration of chromophores. No matter how far the polymer is diluted in terms of the bulk polymer concentration, the concentration of the groups of chromophores along the polymer backbone remains relatively constant [43]. The separation between specific chromophoric groups is determined by the geometry of the chain and flexibility of the bonds and can be on the order 3–8 Å.
This scenario represents an interesting system in which energy can relocate intramolecularly from the initially absorbing chromophore unit to another, often quite some distance away. Intramolecular excitation energy transfer (EET) can occur via (i) ‘hopping’ of excitation energy from one group to another, (ii) movement of excitation energy along the carbon–carbon backbone by an exciton/diffusive mechanism and (iii) movement of excitation energy across loops in a single polymer chain. This energy delocalisation process is often referred to as energy migration.

Guillet defines ‘intramolecular energy migration as any process involving more than one exchange of excitation energy between spectroscopically identical chromophores attached by covalent bonds to a polymer chain’. Energy can also become delocalised over a large portion of a polymer chain, for example in conjugated polymers [44].

The process of energy migration can often be considered as a series of energy transfer hops (steps) between largely identical, weakly coupled chromophores and often modelled in terms of Förster resonance energy transfer. This process is often referred to as ‘homo-FRET’, particularly in the biological literature. In a concentrated solution, or in a polymer or other macromolecule such as dendrimers the energy can migrate significant distances from the origin point (chromophore) of light absorption by undergoing a large number of such steps. Each step can result in the loss of the original photoselected population of absorption transition moments. This can be understood in terms of figure 3. In this figure a small angle is shown between the absorption (solid blue) and emission (dashed red) transition moments of each chromophore, but this is not essential. In (a) the emission transition moment of the ‘donor’ is perpendicular to the absorption transition of the ‘acceptor’ so no transfer can occur. In (b) these transition dipoles are parallel resulting in efficient transfer, but if the ‘acceptor’ chromophore then emitted a photon of fluorescence before a next transfer step, the emission would be depolarised slightly relative to the case where the emission had occurred from the chromophore that absorbed the light originally. In (c) the series of energy transfer steps eventually results in complete depolarisation of the emission. Note that in each step, energy transfer back to the ‘donor’ may also be possible.

Soutar et al [45–48] have made extensive use of steady-state polarisation experiments to show that in several polymers containing vinyl aromatic monomers there is a linear relationship between the degree of polarisation, the mean sequence length, $l_0$, of aromatic species in the copolymer and the mean number of migration steps, $n$. Since a number of energy transfer steps occur and each will results in some decrease in the polarisation of the emission, the fluorescence emission is usually completely depolarised (i.e. $P = 0$) after five to ten energy transfer steps [43]. We have used TRAMs to investigate energy migration in synthetic aromatic polymers including poly(acenaphthalene) [49] in low temperature glass. Despite the lack of rotational mobility in the glass, we observe extremely rapid and complete emission depolarisation reflecting very fast and efficient energy migration. When the ACE chromophores were spaced apart, the rate of energy migration was observed to slow down, as evidenced by the TRAMs signal [49], but still reached complete depolarisation over several hundreds of picoseconds.

As mentioned above, dendrimers represent an intriguing case study for a high local concentration of chromophores, in which the orientation and separation of the chromophores can be reasonably restrained. TRAMs have been applied to the study of various generations of dendrimers of porphyrins in a low temperature glass (to remove the effect of rotational motion) [50]. In the ‘model’ compound (the first generation, single porphyrin unit) the fluorescence
anisotropy signal is time-independent with a magnitude reflecting the $r_0$ of the porphyrin moiety. In low dendrimer generations the TRAMs shows time-dependence that reports on the depolarisation of the emission over several nanoseconds. This reflects the time-scale of the energy hopping between a limited number of chromophores in certain orientations. However, the 5th generation dendrimer, G5P64 (figure 4), exhibits a more complex and interesting emission anisotropy decay (figure 5) that sheds light on both the EET dynamics and the structure of the macromolecule. A very rapid initial anisotropy decay occurs to a value of $r(t) \approx 0.015$ (at $t = 4$ ns) before gradually regaining intensity and levelling-off at $r_\infty \approx 0.018$. These features allude to complex energy transfer dynamics which cannot be easily explained using the models proposed for the lower generation dendrimers. The ‘dip and rise’ behaviour in the $r(t)$ curve suggests that more than one species exists in the system whose contributions to the effective $r(t)$ vary with time. This is easily followed from the polarisation addition law (equation (9)) [51].

The fluorescence decay for G5P64 measured at 77 K was describe satisfactorily only by a triple exponential function with decay times of 0.64 ns, 2.44 ns and 12.5 ns with corresponding pre-exponential fractions of 0.39, 0.27 and 0.34. The two short decay times...
(\(\tau_1\) and \(\tau_2\)) were attributed to relatively strong couplings between porphyrins (i.e. the superradiance effect) whereas the longest decay time component \(\tau_3\), close to the lifetime of the model compound, was assigned to porphyrins with much weaker interchromophoric interactions. Thus, in GSP6, there are in general two species contributing to the effective fluorescence anisotropy signal. The first species (core porphyrins) are closely packed together such that strong couplings are experienced that ultimately give rise to the two short lifetimes and are revealed in the rapid initial anisotropy decay. Their total contributions to \(r(t)\) are important at short times but diminish rapidly (i.e. \(f_1(\infty)r_1(\infty) \to 0\)) with a recovery of the \(r(t)\) intensity at longer times due to the increasing dominance of the \(r(t)\) due to the second contributing species, porphyrins with the long lifetime component (i.e. adventitious porphyrins), at later times. The limiting anisotropy clearly suggests that EET occurs only between four adventitious porphyrins which are too distant from the core chromophores to allow any effective energy transfer.

A simple model was proposed to illustrate the EET dynamics. It was assumed that 56 core porphyrins adopt a circular arrangement with a uniform interchromophore separation of 9 Å \((k = 17.4 \text{ ns}^{-1})\) while each of the four adventitious chromophores are 18 Å \((k = 0.27 \text{ ns}^{-1})\) apart. For the current case, the polarisation addition law (equation (9)) can be rewritten as:

\[
\sum_{j=1}^{N} f_j(t)\tau_j = f_{core}(t)r_{core}(t) + f_{adv}(t)r_{adv}(t)
\]  

(13)

where the fractional contributions to the \(r(t)\) due to the ‘core’ and ‘adventitious’ porphyrins are given by \(f_{core}(t) = \frac{(I_1(t) + I_2(t))}{(I_1(t) + I_2(t) + I_3(t))}\) and \(f_{adv}(t) = \frac{(I_3(t))}{(I_1(t) + I_2(t) + I_3(t))}\) respectively.

The two contributing anisotropy factors \(r_{core}(t)\) and \(r_{adv}(t)\) were computed from the Pauli master equation and the theoretical anisotropy decay obtained from equation (13) was in excellent agreement with the experimental results as shown in figure 5. The analysis of the TRAMs data, therefore led to the proposal that structural defects may be the cause for complex \(r(t)\) data. This system represents a beautiful case study of the effects of energy transfer/migration on the time-dependence of fluorescence anisotropy signals.

4. Excited-state photophysics

We have also observed complex time-resolved fluorescence anisotropy data from two simple aromatic chromophores, acenaphthene (ACE) and (9-phenanthryl)methyl pivalate (9PhMP) in low temperature glasses. [52] Under these conditions rotational motion of the fluorophores should be completely eradicated and the fluorescence anisotropy would be expected to be time-independent (i.e. a constant \(r\) value corresponding to \(r_0\) of the fluorophore). This is not observed and in this case the complex \(r(t)\) behaviour can be attributed to the effects of the interchange of energy between the energy levels in the chromophores dictated by the photophysics of the fluorophore.

4.1. Acenaphthene

The fluorescence from ACE in room temperature solution and in a MTHF glass at 77 K decays over quite similar timescales but its kinetics is excitation wavelength dependent. Naturally, the \(r(t)\) profile of ACE in room temperature solution decays to zero on picosecond timescales characteristic of the rotational motion of the fluorophore. However, the \(r(t)\) profiles obtained from ACE (figure 6) in a solid MTHF glass at 77 K show several intriguing features; (i) the \(r(t)\) profiles are significantly time-dependent over tens of nanoseconds, (ii) the profiles are markedly different for different excitation/emission wavelength

![Figure 6. Time-resolved fluorescence anisotropy profiles of ACE in a low temperature glass.](image)
combinations and (iii) the profile obtained at the excitation/emission wavelengths 295/337 nm changes sign from positive $r$ to negative. The latter point is difficult to reconcile based on a simplistic viewpoint whereby emission depolarisation would be expected to lead to a $r(t)$ value tending to zero rather than changing sign.

The time-resolved fluorescence decay observations, coupled with a steady-state excitation/emission wavelength fluorescence anisotropy plot [52] and the observed complex fluorescence anisotropy behaviour, suggest the cause of the complex $r(t)$ behaviour. The transitions between the excited-state energy levels of a molecule are another pathway able to give rise to complex time-resolved fluorescence anisotropy behaviour if the emission is associated with multiple interconverting excited states. Such a situation has been reported by Fleming et al [53–57] in their investigation of the effects of so-called ‘level kinetics’ on the time-dependent fluorescence polarisation behaviour of tryptophan in solution on picosecond time-scales.

Assuming a relatively simple energy level scheme (figure 7), Fleming et al [53–57] extended the work reported by Andrews and Forster [58] to derive expressions for the time-dependent populations of the two states, ‘level $a$’ and ‘level $b$’ and the resulting time-resolved fluorescence anisotropy, assuming that certain proportions of the two states were initially excited, conversion between the two states could occur and the monitored emission could arise from either or both states. An energy trapping pool (referred to as ‘products’ in figure 7) accounts for any photodegradation or other long-lived (e.g. triplet) states.

Following the terminology of Fleming et al [57] assuming the fluorescence decay time and fluorescence quantum yield of the chromophore are known, the following parameters can be defined in terms of the radiative, $k_{a}^{r}, k_{b}^{r}$ and non-radiative, $k_{a}^{nr}, k_{b}^{nr}$, rate constants, of levels $a$ and $b$, respectively.

\[
k_a = k_a^r + k_a^{nr}
\]
\[
k_b = k_b^r + k_b^{nr}
\]

\[
\delta = k_a - k_b - k_{ab} - k_{ba}
\]

\[
Q = \left[\delta^2 + 4k_{ab}k_{ba}\right]^{1/2}
\]

\[
k = k_a + k_{ab} + k_b + k_{ba}
\]

\[
\lambda_1 = \frac{-(k - Q)}{2}
\]

\[
\lambda_2 = \frac{-(k + Q)}{2}
\]

The excited-state populations of the two states as a function of time are then given by:

\[
K^a(t) = \frac{1}{2Q} \left\{ K^a(0)[Q - \delta] + 2k_{ba}K^b(0) \right\} \exp(\lambda_1t)
\]

\[
+ \frac{1}{2Q} \left\{ K^a(0)[Q + \delta] - 2k_{ab}K^b(0) \right\} \exp(\lambda_2t)
\]

and

\[
K^b(t) = \frac{1}{2Q} \left\{ K^b(0)[Q + \delta] + 2k_{ab}K^a(0) \right\} \exp(\lambda_1t)
\]

\[
+ \frac{1}{2Q} \left\{ K^b(0)[Q - \delta] - 2k_{ba}K^a(0) \right\} \exp(\lambda_2t)
\]

where $K_a(0)$ and $K_b(0)$ are the initial populations of states $a$ and $b$ (i.e. these parameters are proportional to the amount of each state excited initially by the incident laser pulse).

According to this model, the time-dependent anisotropy due to each level is given by:

\[
\frac{r^a(t)K^a(t)}{\cos^{2}\theta_{E}^{a(t)}} = \left(\frac{1}{2Q}\right) \left\{ \left[ K^a(0)P_2(\cos \theta_{E}^{a(t)})[Q - \delta] + 2k_{ba}K^b(0)P_2(\cos \theta_{E}^{b(t)}) \right] \exp[(\lambda_1 - 6D)t]
\]

\[
+ \left[ K^a(0)P_2(\cos \theta_{E}^{a(t)})[Q + \delta] - 2k_{ab}K^b(0)P_2(\cos \theta_{E}^{b(t)}) \right] \exp[(\lambda_2 - 6D)t] \right\}
\]
It is known that the fluorescence anisotropy is given by:

\[
r(t) = \frac{k_{g_{a}(w)}r^{(a)}(t)K^{(a)}(t) + k_{g_{b}(w)}r^{(b)}(t)K^{(b)}(t)}{[K^{(a)}(t)k_{g_{a}(w)} + K^{(b)}(t)k_{g_{b}(w)}]}
\]

where \(k_{g_{a}(w)}\) and \(k_{g_{b}(w)}\) are the spectral lineshape functions which define the proportion of the monitored emission emanating from level \(a\) or \(b\) respectively, following excitation of various proportions of the two levels as given by \(K_{a}(0)\) and \(K_{b}(0)\).

Finally, the expression for the total fluorescence anisotropy is given by:

\[
r(t) = \frac{1}{4} \left( 1 + 3 \cos^2 \theta^{(a)}_E \right) + 2k_{ab}\cos \theta^{(b)}_E + \frac{1}{2} \exp \left[ (\lambda_s - 6D) rt \right]
\]

where \(P_2(\cos \theta^{(b)}_E) = \frac{1}{4} \left( 1 + 3 \cos^2 \theta^{(b)}_E \right)\) terms correspond to the second Legendre polynomial [59] and take into account the angles between the various absorption and emission transition dipoles of the two levels, namely, \(\theta^{(a)}_E, \theta^{(b)}_E, \theta^{(a)}_A\) and \(\theta^{(b)}_A\).

Finally, the expression for the total fluorescence anisotropy is given by:

\[
r(t) = \frac{1}{2} \left( k_{g_{a}(w)}r^{(a)}(t)K^{(a)}(t) + k_{g_{b}(w)}r^{(b)}(t)K^{(b)}(t) \right)
\]

where \(k_{g_{a}(w)}\) and \(k_{g_{b}(w)}\) are the spectral lineshape functions which define the proportion of the monitored emission emanating from level \(a\) or \(b\) respectively, following excitation of various proportions of the two levels as given by \(K_{a}(0)\) and \(K_{b}(0)\).

We have published [52] an analysis of the ACE data of figure 6 based on the model proposed by Fleming et al [57]. The three \(r(t)\) decays were fit ‘globally’ i.e. all three curves were fit simultaneously with the various parameters in common as they were optimised using non-linear least squares curve fitting to minimise a ‘global’ chi-squared value calculated over the data from all three decays. The results of applying this model to the ACE \(r(t)\) data are shown as the solid lines in figure 6. The results led to estimates for the rate of conversion from level \(b\) to \(a\), the spectral lineshape functions \(g_{a}(w)\) and \(g_{b}(w)\) and the angles between the absorption and the emission transition dipoles within the fluorophore.

4.2. (9-phenanthryl)methyl pivalate

The observed behaviour discussed above is not restricted to ACE, but we suspect may be observed in many simple aromatic chromophores under the appropriate conditions. For example, we have seen very similar behaviour from (9-phenanthryl)methyl pivalate (9PhMP, inset figure 8). 9PhMP was synthesised as a model compound for energy migration studies in poly((9-phenanthryl)methyl methacrylate), a polymer in which energy migration might be expected to be efficient due to the much reduced excimer formation reportedly exhibited by phenanthryl groups compared with naphthalene derivatives. The time-resolved fluorescence anisotropy from 9PhMP in MTHF glass at 77 K (figure 8) is seen to decay on a nanosecond time scale, have an emission wavelength dependence and change sign under certain excitation/emission wavelength conditions, in behaviour reminiscent to that reported for ACE.

This suggests that the analysis discussed for ACE may also be applicable to 9PhMP, however, the analysis is further complicated, compared to that of ACE, by more complex (triple) exponential fluorescence decay observed from 9PhMP. Analysis of the fluorescence anisotropy decays as a function of emission wavelength, based on the same two-level system under discussion in the previous case, provided fits of comparable quality to those reported for ACE and returned a value for \(k_{ba}\) very similar to that recovered for ACE and the 2,7-dimethoxyxcarbazoles reported by Ganguly et al [60].

These examples, ACE and 9PhMP in low temperature glasses, show how TRAMs can be used to elucidate photophysical behaviour inherent in the fluorophore in the absence of molecular rotation, but also they show how such measurements must be used judiciously when interpreting behaviour exhibited by more complex systems such as polymers. In the final section we show an example where multiple effects might influence the temporal behaviour of fluorescence anisotropy.

5. Behaviour near an interface

Total internal reflection of light propagating through a material of one refractive index that forms an interface with a second material of lower refractive index, at an incident angle larger than the critical angle (defined by Snell’s Law) produces a non-propagating evanescent field within the medium of lower refractive index. This field strength decays exponentially with distance from the interface and can be used to excite and hence generate emission from, fluorophores adsorbed to, or preferentially those in close proximity to, the interface. The use of evanescent-wave excitation affords a number of desirable experimental benefits. One of these that we have exploited is the ability to photoselect preferentially, molecules with a vector component of their absorption transition moment oriented either parallel or perpendicular to the plane of the interface.

We have performed time-resolved fluorescence polarisation measurements of fluorophores near interfacial regions, following evanescent wave excitation. In these experiments, \(s\) (vertically) and \(p\) (horizontally) polarised excitation light was used to generate, sequentially, a series of fluorescence decay profiles corresponding to all four permutations of the excitation and emission polarisation orientations, \(I_{XY}\) (where X and Y correspond to the polarisation of the excitation radiation and the emission, respectively, relative to an interface in the X–Y plane)). These decays were collected at a particular angle of incidence (penetration depth) and the time-dependent ‘in-plane’ and ‘out-of-plane’ anisotropy functions, defined in equations (26) and (27) [61], respectively, calculated.

\[
r_{p}(t) = \frac{I_{YY}(t) - I_{XX}(t)}{I_{YY}(t) + I_{XX}(t)}
\]
Fluorescent molecules are often embedded in a polymer film in order to immobilise them, for example for single molecule spectroscopic studies. This is premised on the assumption that the polymer matrix is highly rigid thereby inhibiting any depolarisation of its emission due to rotational motion and also that the matrix does not alter the photophysics of the fluorophore appreciably. It was for just this reason that we studied the common and well-characterised fluorophore, acridine, doped into a thin film of poly(acrylic acid) (PAA) using polarised evanescent wave-induced fluorescence (p-TREWIF) measurements.

\[
\rho(t) = \frac{I_{2Y}(t) - \frac{1}{2}(I_{YY}(t) + I_{XX}(t))}{I_{2Y}(t) + I_{YY}(t) + I_{XX}(t)}
\]

Figure 8. (a) Time-resolved fluorescence anisotropy profiles and (b) steady-state polarised emission spectrum of 9PhMP in low temperature MTHF glass following excitation at 295 nm.

Fluorescent molecules are often embedded in a polymer film in order to immobilise them, for example for single molecule spectroscopic studies. This is premised on the assumption that the polymer matrix is highly rigid thereby inhibiting any depolarisation of its emission due to rotational motion and also that the matrix does not alter the photophysics of the fluorophore appreciably. It was for just this reason that we studied the common and well-characterised fluorophore, acridine, doped into a thin film of poly(acrylic acid) (PAA) using polarised evanescent wave-induced fluorescence.

Figure 9. Complex \( r(t) \) profiles from p-TREWIF measurements of acridine.
(p-TREWIF) measurements. In search of a suitable method of determining the ‘$G$’-factor under polarised, evanescent wave excitation conditions, we collected time-resolved fluorescence anisotropy profiles from this dye under these conditions expecting to see a time-independent value of $r$, from which the $G$-factor could be calculated by comparison with the known value of $r_0$ for this fluorophore. Instead of a constant value of $r(t)$, we unexpectedly observed complex time-resolved fluorescence anisotropy behaviour (figure 9) [62].

It is clear that the $r(t)$ shows intriguing behaviour. Firstly, the $r(t)$ profiles are time-dependent, which as discussed above is not uncommon. However, these $r(t)$ profiles are also dependent on the plane relative to the interface that is being probed and they also depend somewhat on the solvent from which the polymer film was cast. The interpretation of these data is extremely difficult to reconcile. One interpretation is that the fluorophores might not in fact be held rigidly within the polymer matrix, but rather can experience some degree of rotational motion that differs in the two planes relative to the interface, possibly due to some solvent being retained within the internal structure of polymer matrix. We have managed to model these data using the approach from section 2. These data might also be interpretable in terms of the photophysics of the fluorophore discussed above (section 6), as acridine is known to have an electronic energy level structure similar to that shown in figure 7 [63]. It is also possible that more than one of the above factors can contribute to the observed time-dependent fluorescence anisotropy behaviour; some rotational mobility in addition to some contribution of the photophysics resulting from the close-lying excited-state energy levels.

6. Conclusions

Time-resolved fluorescence anisotropy measurements provide a powerful method to investigate molecular dynamics. However TRAMS data can become complex, even for relatively ‘simple’ scenarios and in this review we have illustrated the value of examining the anisotropy time decay profile data as a first step so complexities become apparent before implementing kinetic modelling and fitting. Initially unexpected complex temporal dependence can be modelled in many cases using the approaches outlined in this review. For the cases described the time-scale of the dynamics were significantly greater than the instrument response function of the experiments. Thus instrumental effects such as the width and substructure of the instrument’s response function (IRF) could be neglected in the analysis. However it should be noted that where this is not the case (i.e. where the time scale of the anisotropy decay is of the same order as the instrument IRF) then more computationally intensive convolution of the IRF with the trial function is required.

In this review we have illustrated with examples from our laboratories that in addition to molecular rotational motion, intrinsic molecular photophysics and energy migration can also give rise to complex temporal dependence of TRAMS signals. Other situations can no doubt lead to additional complexities in anisotropy decay data, however the examples presented in this review highlight both the care required in interpreting anisotropy measurements and also the wealth of molecular and dynamic information that can be revealed by appropriate treatments of the data.

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