Fluorescence Polarization and Anisotropy

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Symbols

H_H	intensity of horizontal emission from horizontal excitation
H_V	intensity of horizontal emission from vertical excitation
I_T	total emission intensity
M	molar mass
$p(\lambda)$	fluorescence polarization at wavelength λ
$r(\lambda)$	fluorescence anisotropy (at wavelength λ)
r_0	intrinsic anisotropy
t	time
V_H	intensity of vertical emission from horizontal excitation
V_V	intensity of vertical emission from vertical excitation
η	viscosity
$\kappa(\lambda)$	V_H/H_H correction factor
τ	fluorescence lifetime
ϕ	rotational correlation time

Fluorescence polarization techniques have proved to be a very useful method of investigating the environment and motion of fluorescent molecules and fluorophores within larger macromolecules.

When molecules are illuminated by plane-polarized light, those with their transition electric dipole moment parallel with the plane of polarization will preferentially absorb the light ('photoselection'). Subsequent emission of light from this excited electronic state, as the molecule returns to the original electronic ground state, will be similarly polarized parallel to the transition moment of each molecule. If the molecules are unable to rotate during the lifetime of the excited state, then the plane of polarization of the emitted light will be consistent and directly related to that of the exciting light (their electric vectors will be parallel). However, if the molecules are able to rotate within this time period, then the degree of polarization will be diminished. A similar description can be envisaged for fluorophoric groups within larger molecules; in this case, the rotational relaxation involves the tumbling of the complete molecule together with the localized motion of the fluorophore with respect to the molecular environment.

Although rotational relaxation is the main mechanism for loss of polarization, other phenomena can also contribute to depolarization, not least radiationless transfer among fluorophores. Furthermore, if the emission occurs between a different pair of electronic states from that of the original absorption, with differently oriented transition moments with respect to the molecular axes, then a change in polarization may be observed.

Steady-State Anisotropy

In a typical fluorescence spectrometer, the excitation beam illuminating the sample and the optical path employed to detect the emission are orthogonal to each other and define a 'horizontal' plane. By employing linear polarizers before and after the sample, the plane of polarization of the excitation beam can be controlled and the polarization components of the emitted light determined. Specifically, by orientating the polarizers as either parallel or perpendicular to the horizontal plane, the plane of polarization of the excitation or detected emission can be described as either parallel to the plane (H, for horizontal) or perpendicular to it (V, for vertical). Four emission spectra can then be acquired:

V_V = intensity of vertical emission from vertical excitation
H_V = intensity of horizontal emission from vertical excitation
V_H = intensity of vertical emission from horizontal excitation
H_H = intensity of horizontal emission from horizontal excitation.

In these circumstances, for an *ideal* instrument, the *fluorescence polarization*, $p(\lambda)$, for a sample at a given wavelength λ is

$$p(\lambda) = \frac{(V_V - H_V)}{(V_V + H_V)} \quad [1]$$

and the corresponding *fluorescence anisotropy*, $r(\lambda)$, as

$$r(\lambda) = \frac{(V_V - H_V)}{(V_V + 2H_V)} \quad [2]$$

In principle, this would allow the fluorescence polarization or anisotropy to be determined with just two spectral acquisitions: V_V and H_V. However, in practice, it is vital to correct for the differing efficiencies of the emission monochromator and detector toward vertically and horizontally polarized light, which may be substantial. Consequently, for isotropic samples, eqn [1] can

be amended to

$$p(\lambda) = \frac{(V_V - \kappa(\lambda)H_V)}{(V_V + \kappa(\lambda)H_V)} \quad [3]$$

where

$$\kappa(\lambda) = V_H/H_H \quad [4]$$

and similarly for the fluorescence anisotropy,

$$r(\lambda) = \frac{(V_V - \kappa(\lambda)H_V)}{(V_V + 2\kappa(\lambda)H_V)} \quad [5]$$

The basis for the correction ratio κ is that, for horizontally polarized excitation of isotropic samples, the resulting vertically and horizontally polarized fluorescences detected should be of equal intensity; any difference may be attributed to the polarization dependence of the emission monochromator and detector.

The fluorescence polarization (p) and anisotropy (r) formalisms essentially embody the same information and each has its virtues and applications. In the realms of chemical and biochemical phenomena, where multiple fluorophores may be present, anisotropy has the advantage in possessing a linear relationship with mixture composition; the mean anisotropy of a mixture of species with fractional intensities f_i and individual anisotropies r_i is given by simply the sum of their products.

$$\bar{r} = \sum_{i}^{\text{Fluo.}} f_i r_i \quad [6]$$

Nonetheless, the polarization and anisotropy can be readily interconverted where necessary via

$$p = \frac{3r}{2 + r} \quad [7]$$

$$r = \frac{2p}{3 - p} \quad [8]$$

The fluorescence polarization $p(\lambda)$ and anisotropy $r(\lambda)$ both take the value of unity where there is a complete preservation of polarization during the excited-state lifetime (e.g., if the molecules are immobile and aligned for maximal absorption). Likewise, they both take the value of zero if there is a complete loss of polarization (i.e., a fast randomization of orientation). However, their values differ for cases between these extremes. In principle $-1 < p < 1$ and $-0.5 < r < 1$, although the limits are rarely encountered; negative values may be indicative of processes beyond depolarization through rotational relaxation, such as transitions to other excited states. In the case of an isotropic, randomly orientated distribution of molecules, the consequences of photoselection dictate that the maximal anisotropy is $r = 0.4$ (corresponding to $p = 0.5$).

If the only significant process for depolarization is rotational relaxation, then the fluorescence anisotropy, for a single molecule or fluorophore, may be given by a form of the Perrin equation:

$$r = \frac{r_0}{(1 + \tau/\phi)} \quad [9]$$

where τ is the fluorescence lifetime, ϕ is the rotational correlation or relaxation time for the fluorophore's tumbling, and r_0 is the intrinsic anisotropy in the absence of rotational relaxation. Small molecules (less than 1000 Da molecular mass) with fluorescence lifetimes of the order of $\tau = 10$ ns are able to tumble with relative ease in low-viscosity solvents (e.g., water), typically $\phi = 0.1$ ns and thus $\tau \gg \phi$, and there is almost complete depolarization during the fluorescence lifetime ($r \rightarrow 0$). In contrast, a fixed fluorophore in a large protein may have the same fluorescence lifetime, but a rotational relaxation time of $\phi = 10$ ns, so that $\tau \sim \phi$ and there is significant preservation of the polarization.

As a convenient rule of thumb, the rotational relaxation time (in nanoseconds) for a spherical molecule of mass M (g mol^{-1}) in a medium of viscosity η ($\text{g cm}^{-1}\,\text{s}^{-1}$) is

$$\phi \approx 0.03\,\eta M \text{ ns} \quad [10]$$

with, for example, $\eta = 0.01\ \text{g cm}^{-1}\,\text{s}^{-1}$ for water.

In the case of a small fluorescent molecule binding to a large macromolecule, the bound form should be distinguishable from the unbound form by having a substantially greater fluorescence anisotropy. Likewise, fluorophores rigidly held within a macromolecule would show significantly greater anisotropies than those exposed to the solvent and possessing some degree of motional freedom. Thus, one can employ fluorescence anisotropy to locate groups within a structure. This can prove particularly powerful if an environmental change (including perhaps the binding of a compound) induces a change in rigidity about observable fluorophores. Finally, if the variation in fluorescence anisotropy is plotted against wavelength, spectroscopic bands originating from distinct transitions or fluorophores may be distinguished by their differing anisotropies.

Fluorescence Lifetime Anisotropy

By the use of fluorescence lifetime instrumentation, one can further determine the evolution of the fluorescence anisotropy with time during and beyond the lifetime of the excited state. In the simplest case of a fluorophore in solution, with a single fluorescence lifetime and

depolarization through rotational relaxation alone, the fluorescence anisotropy will decay according to

$$r(t) = r_0 \exp(-t/\phi) \quad [11]$$

whereas the total emission intensity, $I_T(t)$, will decay as

$$I_T(t) = I_T(t_0)\exp(-t/\tau) \quad [12]$$

Thus, the rotational relaxation and fluorescence lifetime may be directly distinguished.

In more complex cases, perhaps involving binding kinetics or multiple environments, it is possible to deduce characteristic half-lives for the various processes. For example, one may identify subpopulations of molecules that are bound in different modes and even characterize the residual motion of a molecule while it is bound to a site that is itself tumbling. Nonetheless, these complex applications all require a sufficiently robust hypothesis regarding the phenomena being modeled if the interpretations are to be valid.

See also: Chiroptical Spectroscopy, Oriented Molecules and Anisotropic Systems, Fluorescence Theory, NMR in Anisotropic Systems, Theory, UV-Visible Fluorescence Spectrometers.

Further Reading

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