Fluorescence Spectroscopy

Molecular luminescence spectroscopy is the measurement of light emitted by a sample. Luminescence is the emission of light in the visible or ultraviolet region where the sample is not being excited by heating. In luminescence, the light is emitted as the electronically excited molecule returns to the ground state. The excited state can be generated by a variety of techniques; thus luminescence is divided into categories depending on the mechanism by which the excited state was formed. In chemiluminescence, the excited state is generated by a chemical reaction; in **photoluminescence**, the transition to the excited state is the result of absorption of electromagnetic radiation.

The usefulness of fluorescence spectroscopy stems from two main factors. First, the majority of molecules do not exhibit fluorescence. As a result, in most samples, the background fluorescence will be very small, and therefore the technique can detect specific molecules with high sensitivity. Second, the spectral properties of the fluorescence emission are usually highly sensitive to local environment. While for the study of small molecules this is not especially useful, because it merely measures changes in the solvent, for fluorophores in macromolecules, the local environment is generally different in different locations within the macromolecule, and therefore fluorescence allows direct monitoring of changes in conformation or interactions with other molecules.

Fluorescence Quantum Theory

Recall from the discussion of absorbance that:

$$\frac{-dI(\nu)}{dt} = h\nu\left(N_a B_{ab} - N_b B_{ba}\right)I(\nu)$$

This suggests that molecules should continue a net absorption of light until $N_a = N_b$. This is illogical, since the molecules would be expected to be in the ground state in the absence of light. The implication is that molecules must have alternate methods of returning to the ground state other than by stimulated emission.

Based on statistical mechanics, in the absence of light the ratio of molecules in the ground to the excited states should be:

$$\frac{N_a}{N_b} = e^{-\left(\frac{E_a - E_b}{kT}\right)} = e^{+\frac{h\nu}{kT}}$$

Einstein pointed out that stimulated emission was not the only method for return to the ground state, and that the molecule could go from S_b to S_a, by spontaneous emission, A_{ba} At equilibrium:

$$\frac{N_a}{N_b} = \frac{B_{ba}I(\nu) + A_{ba}}{B_{ab}I(\nu)}$$

If $B_{ab} = B_{ba}$, then: $\frac{N_a}{N_b} = 1 + \frac{A_{ba}}{B_{ab} I(\nu)}$

The radiation density for a black body is: $I(\nu) = \frac{8\pi h\nu^3}{c^3(e^{h\nu/kT} - 1)}$

At equilibrium, the N_a / N_b ratios for the statistical mechanics equation and the spontaneous emission equations are equal. Then:

$$\frac{N_a}{N_b} = 1 + \frac{A_{ba}}{B_{ab} I(\nu)} = \frac{A_{ba} c^3 (e^{h\nu/kT} - 1)}{B_{ab} 8\pi h\nu^3}$$

and therefore (using the N_a / N_b ratios from the statistical mechanics equation:

$$A_{ba} = 8\pi h\nu^3 c^{-3} B_{ab}$$

Because: $B_{ab} = \frac{2}{3}\frac{\pi}{\hbar^2} D$, it follows that: $A_{ba} = \frac{32\pi^3\nu^3}{3c^3\hbar} D$

The dipole strength D and the frequency can be measured. In principle, therefore the rate of spontaneous emission should be:

$$\frac{dN_b}{dt} = -A_{ba} N_b$$

Integrating gives:

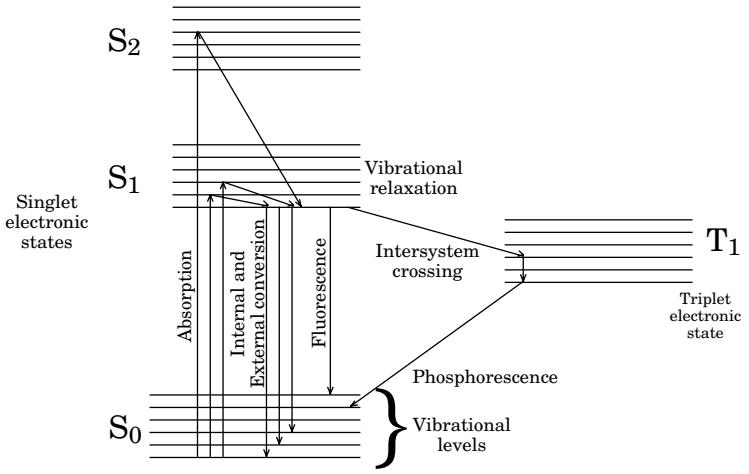
$$N_b(t) = N_b(0)^{-A_{ba}t}$$

The radiative lifetime of state S_b can therefore be defined as $\tau_R = 1/A_{ba}$. The relationship between D and A_{ba} predicts that extinction coefficients should be inversely correlated to excited state lifetimes; this means that molecules with strong absorption bands should have short excited state lifetimes, and are somewhat more likely to fluorescence than molecules with smaller extinction coefficients. Observation generally supports this prediction for molecules where the same initial excited state also emits the radiation. For most fluorescence molecules, typical values observed for τ_R are 10^{-6} to 10^{-9} seconds. For molecule with extinction coefficients greater than 10^3 $(M \bullet cm)^{-1}$, excited state lifetimes are typically 10^{-7} to 10^{-9} seconds, while molecules with lower extinction coefficients have longer lifetimes.

Absorption and Emission Processes

A number of changes in electronic and vibrational states may occur during absorption and emission processes. As with most processes in chemistry and

physics, the overall kinetics of the process can be important in determining which processes will be favored. The Jablonski diagram (see below) is useful for illustrating these processes; a full consideration of relative likelihoods of different processes involves an understanding of the time scales involved and of the factors that influence the relative probabilities of the different transitions that return the molecule to the ground state.



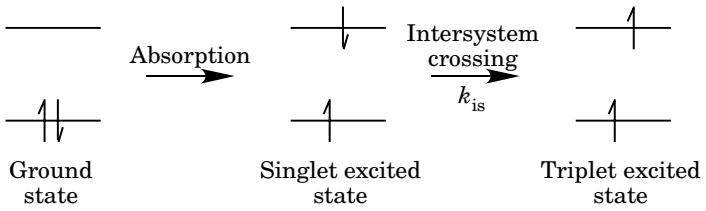
The absorption of a photon, with concomitant change in electronic state (the upward pointing arrows in the Jablonski diagram above) is very fast (10^{-15} seconds). In most cases, the result is a molecule that is in an excited vibrational state as well as an excited electronic state. Vibrational relaxation is fast (10^{-12} seconds), and usually occurs via collisions. Because vibrational relaxation within the same electronic state is faster than electronic relaxation from the S_1 to the S_0 state, vibrational relaxation generally occurs before the return to the ground (S_0) state.

For most molecules, the ground state is a singlet state. During absorption and emission processes, singlet-to-singlet transitions are more likely than singlet-to-triplet transitions. Depending on the energy of the absorbed photon, the molecule may transition from the ground state to different excited states (S_1, S_2, or higher). However, in the vast majority of molecules, fluorescence only occurs as a process of transition from S_1 to S_0. This means that, even if the molecule were excited to S_2 or higher, it will generally drop to S_1 by non-radiative processes before fluorescing. This has obvious consequences for the wavelength range over which the fluorescence emission will be observed. The rate constant for fluorescence (k_F) is a numerical value related to the likelihood of fluorescence emission.

As shown in the Jablonski diagram above, non-radiative processes compete with fluorescence.

If the S_1 state has a long lifetime, the molecule may undergo **intersystem crossing**, which is transition to the triplet state (this is a forbidden process, but may occur at appreciable rates for some molecules; the process is favored because the T_1 state is lower in energy than the S_1 state). Return to the ground state via phosphorescence is very slow compared to the other processes (10^{-4} to 10^{4} seconds), because triplet-to-singlet transition is also a forbidden process. While

phosphorescence involves photon emission, the triplet-to-singlet transition may also occur by non-radiative processes (especially in solution.) In solution, phosphorescence is rarely observed unless oxygen (an effective quenching agent for both triplet and singlet states) is removed from the solution. In most biochemical experiments, only fluorescence is useful, because the fluorescent lifetime is short enough to allow fluorescence to occur before quenching. Even if phosphorescence does occur, the rate constant for the process is very small; only the rate constant for intersystem crossing (abbreviated k_{is}) usually matters for consideration of fluorescence.



Internal conversion is a non-radiative process that allows the molecule to return to the ground state via vibrational relaxation or other relaxation processes within the molecule. Internal conversion is temperature dependent, which complicates the use of fluorescence to monitor other temperature dependent processes (especially thermal unfolding of proteins). The rate constant for internal conversion is abbreviated k_{ic}.

External conversion is a non-radiative process that allows the molecule to return to the ground state as a result of collisions with solvent molecules. The rate constant for internal conversion is abbreviated k_{ec}.

Quenching is related to external conversion; in quenching, solute molecules remove the extra energy from the excited state. Although quenching is (at least) a second order process, the high concentration of quenching agent required for most types of quenching allows a pseudo-first order analysis of the rate constant k_q; the rate for the process depends on both the rate constant and on the quencher concentration [Q]. Fluorescence quenching can be an experimentally useful phenomenon, and is discussed in more detail below.

The last type of non-radiative process is photodecomposition, in which the excited state undergoes a chemical change to a different molecule, and never returns to the ground state of interest. The rate constant for photodecomposition is abbreviated k_{pd}.

The process with the largest rate of relaxation will predominate, but if several processes have similar rates, the processes will compete. The **quantum yield** ϕ_F is the fraction of absorbed light that is reemitted as fluorescent photons:

$$\phi_F = \frac{\#\ \text{of molecules that luminesce}}{\text{total}\ \#\ \text{of excited molecules}} = \frac{k_F}{k_F + k_{ic} + k_{ec} + k_{is} + k_{pd} + k_q[Q]}$$

The kinetics of the return to the ground state is:

$$\frac{-d[S_b]}{dt} = (k_F + k_{ic} + k_{ec} + k_{is} + k_{pd} + k_q[Q])[S_b]$$

Integrating gives:

$$S_b(t) = S_b(0)\, e^{-\frac{t}{\tau_F}}$$

In this equation, τ_F is the observed lifetime of the fluorescent state as a result of all of the processes that allow decay of the excited singlet state.

$$\tau_F = \frac{1}{k_F + k_{ic} + k_{ec} + k_{is} + k_{pd} + k_q[Q]}$$

The quantum yield is therefore:

$$\phi_F = \frac{\tau_F}{\tau_R}$$

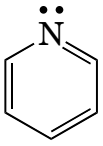
Fluorophore properties

Fluorescent molecules, and fluorescent functional moieties within larger molecules are called **fluorophores**. In general, a fluorophore is likely to contain an extensive conjugated π system.

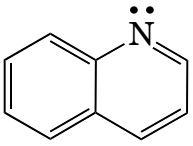
Absorption bands corresponding to $n \rightarrow \pi^*$ transitions usually involve small extinction coefficients, and therefore long lifetimes; these systems usually have small quantum yields, because the non-radiative processes tend to dominate.

Absorption bands corresponding to $\pi \rightarrow \pi^*$ transitions usually involve large extinction coefficients, and therefore shorter lifetimes; these systems are more likely to exhibit fluorescence, because k_F is usually relatively large.

As examples, consider the two molecules below.



Pyridine



Quinoline

Pyridine has non-bonding electrons on the heteroatom. For pyridine intersystem crossing and other non-radiative processes tend to dominate. Quinoline, which has a larger π system, is more strongly fluorescent.

Fluorescence intensity

The number of excited molecules is proportional to the decrease in light intensity that occurs during passage of the light through the sample. This intensity change can be calculated from the Beer-Lambert law:

$$I = I_0 e^{-\ln(10)\varepsilon_\lambda cl}$$

where ε_λ is the extinction coefficient at the exciting wavelength used. For low absorbance values, this can be expanded to:

$$I = I_0[1 - \ln(10)\varepsilon_\lambda cl]$$

The emission intensity F_λ for one type of molecule at a given wavelength is a function of the quantum yield ϕ_F, the fraction of emission that occurs at that wavelength f_λ, and the fraction of the radiation that is actually collected by the detector, j:

$$F_\lambda = \ln(10)\ \varepsilon_\lambda\ c\ l\ I_0\ \phi_F\ f_\lambda j$$

Thus, the fluorescent intensity is related to the probability that the solution will absorb light of the wavelength used (from the $\varepsilon_\lambda c$ term) and the probability that the molecule will emit light at a particular wavelength.

An alternative equation for fluorescence intensity can be derived as follows:

$$F_\lambda = k'(I_0 - I)$$

where k' is a rate constant that for the fluorescence process that takes quantum yield into account, I_0 is the incident radiation, and I is the remaining intensity exiting the sample (the difference $I_0 - I$ is thus the amount of absorbed light).

Because $A = \varepsilon lc = \log\left(\frac{I_0}{I}\right)$, then $F_\lambda = k' I_0(1-10^{-\varepsilon lc})$

This equation contains a concentration term in the exponent. It can be expanded in the McLaurin series:

$$F_\lambda = k' I_0\left(\ln(10)\varepsilon lc - \frac{\left(\ln(10)\varepsilon lc\right)^2}{2!} + \frac{\left(\ln(10)\varepsilon lc\right)^3}{3!} \ldots\right)$$

Note that fluorescence is not really linearly related to concentration. (In actual experimental systems, absorbance also tends to deviate from linear behavior, but theory states that it should be linear, while fluorescence is theoretically inherently

non-linear.) Higher I_0 (excitation source intensity) yields greater fluorescence. In general, higher concentration, within some limits, is also associated with greater fluorescence.
If $\ln(10)\varepsilon lc < \sim 0.05$, then the higher order terms become less important. Therefore, at low concentrations, the fluorescence intensity approaches a linear expression:

$$F_\lambda = k' I_0 \left(\ln(10)\varepsilon lc\right) = Klc$$

High concentrations can result in self-quenching, inner filtering and other artifacts. Obtaining a reasonably linear fluorescence response as a function of concentration is another reason for maintaining a concentration that yields an absorbance of <0.1.

In principle, one of these fluorescence intensity equations could be used to calculate the quantum yield for a molecule. In practice, because of a variety of artifacts inherent in fluorescence instruments, however, relative quantum yields are measured. The method involves using standards such as quinine sulfate in 0.5 M H_2SO_4 (ϕ_F = 0.7) or fluorescein in 0.1 M NaOH (ϕ_F = 0.93), at concentrations such that εlc is the same for the standard and the unknown.

