Application of the Lippert Equation

The emission spectra of many fluorophores used to label macromolecules are known to be sensitive to solvent polarity. One of the best-known examples is the probe ANS. This class of probes has become widespread since its introduction in 1954. ANS and similar molecules are essentially nonfluorescent when in aqueous solution, but become highly fluorescent in nonpolar solvents or when bound to proteins and membranes. These probes are highly sensitive to solvent polarity and can potentially reveal the polarity of their immediate environments. For example, the emission maximum of 2,6-ANS shifts from 416 nm in acetonitrile to about 460 nm in water (Figure 1), and the emission maximum could be used to estimate the polarity of the binding site of ANS on the macromolecules. Another reason for the widespread use of these probes is their low fluorescence in water. For example, the quantum yield of 1-anilinonaphthalene-8-sulfonate (1,8-ANS) is about 0.002 in aqueous buffer, but near 0.4 when bound to serum albumin. This enhancement of the quantum yield is useful because the fluorescence of a dye–protein or dye–membrane mixture results almost exclusively from the dye that is bound to the biopolymers, with almost no contribution from the unbound probe. The solvent sensitivity of a fluorophore can be estimated by a Lippert plot. This is a plot of $(\bar{\nu}_A - \bar{\nu}_F)$ versus the orientation polarizability (Δf). The most sensitive fluorophores are those with the largest change in dipole moment upon excitation. Representative Lippert plots for two naphthylamine derivatives are shown in Figure 2. The sensitivity of these fluorophores to

solvent polarity is probably due to a charge shift from the amino group towards the electronegative sulfonic acid group.

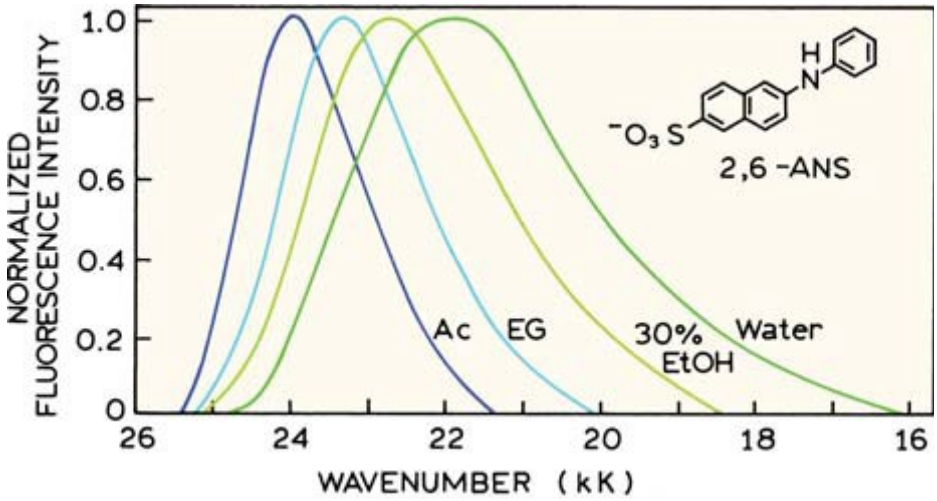

Figure 1. Normalized emission spectra for 6-anilino-2-naphthalene sulfonic acid (ANS). The solvents are acetonitrile (Ac), ethylene glycol (EG), 30% ethanol/ 70% water (30% EtOH), and water.

The N-phenyl-N methyl derivative of 6-aminonaphthalene-2-sulfonic acid is more sensitive to solvent polarity than the unsubstituted amino derivatives. This higher sensitivity to solvent polarity is probably because the phenyl ring allows for a larger charge separation than the unsubstituted amino group. The linearity of these plots is often regarded as evidence for the dominant importance of general solvent effects in the spectral shifts. Specific solvent effects lead to nonlinear Lippert plots . The data in Figure 2 are for a limited range of similar solvents. Specifically, these were ethanol–water mixtures, so the same specific effects due to hydrogen bonding were present in all mixtures. In general, the attachment of side chains to the amino group, especially aromatic groups, enhances the sensitivity to solvent polarity. The change in dipole moment is large when electron donating alkyl groups are attached to the

nitrogen. Attachment of a toluyl group further increases the charge separation in the excited state.

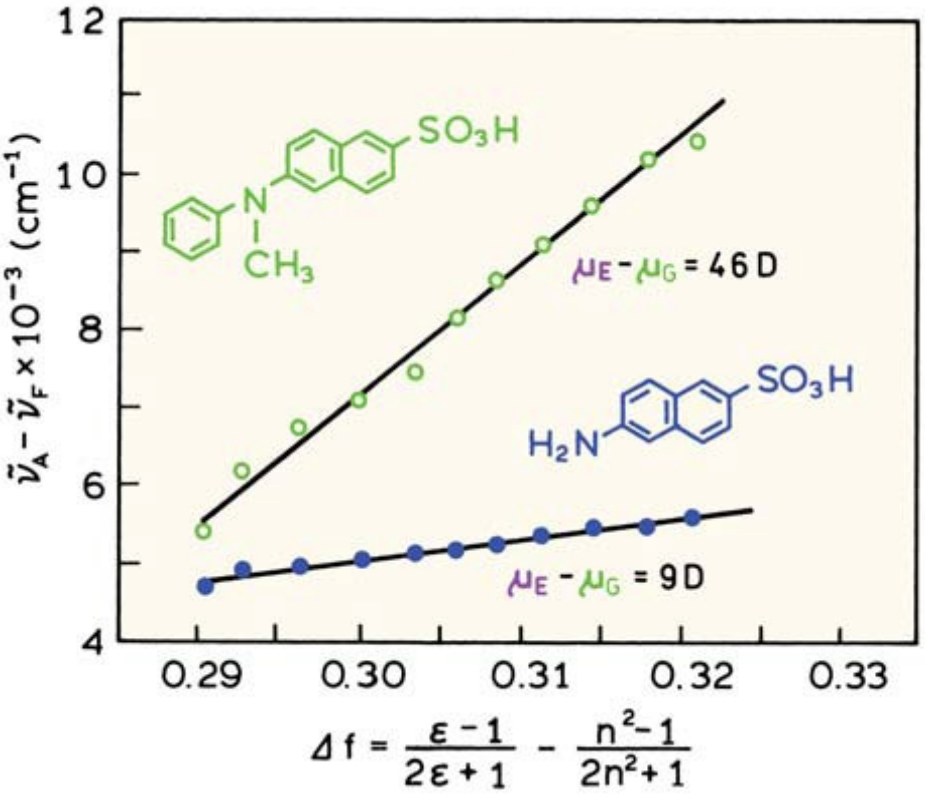

Figure 2. Lippert plots for two naphthyl amine derivatives in ethanol–water mixtures. Data are shown for N-phenyl-N-methyl-6-aminonaphthalene-2-sulfonate (○) and 6-aminonaphthalene-2-sulfonate (•).

SPECIFIC SOLVENT EFFECTS

we described the general interactions between fluorophores and solvents, as modeled by the Lippert equation. These general effects are determined by the electronic polarizability of the solvent (which is described by the refractive index *n*) and the molecular polarizability (which results from reorientation of

solvent dipoles). The latter property is a function of the static dielectric constant, Ɛ. In contrast, specific interactions are produced by one or a few neighboring molecules, and are determined by the specific chemical properties of both the fluorophore and solvent. Specific effects can be due to hydrogen bonding, preferential solvation, acid–base chemistry, or charge-transfer interactions. The spectral shifts due to such specific interactions can be substantial, and if not recognized, limit the detailed interpretation of fluorescence emission spectra. Specific solvent–fluorophore interactions can often be identified by examining emission spectra in a variety of solvents. Typical data for 2-anilinonaphthalene (2-AN) in cyclohexane are shown in Figure 3. Addition of low concentrations of ethanol, which are too small to alter the bulk properties of the solvent, result in substantial spectral shifts. Less than 3% ethanol causes a shift in the emission maximum from 372 to 400 nm. Increasing the ethanol concentration from 3 to 100% caused an additional shift to only 430 nm. A small percentage of ethanol (3%) caused 50% of the total spectral shift. Upon addition of the trace quantities of ethanol one sees that the intensity of the initial spectrum is decreased, and a new red-shifted spectrum appears. The appearance of a new spectral component is a characteristic of specific solvent effects. It is important to recognize that solvent-sensitive fluorophores can yield misleading information on the polarity of their environments if specific interactions occur, or if solvent relaxation is not complete. Because the specific spectral shift occurs at low ethanol concentrations, this effect is probably due

to hydrogen bonding of ethanol to the amino groups, rather than general solvent effects.

Another example of specific solvent effects is provided by 2-acetylanthracene (2-AA) and its derivatives. Emission spectra of 2-AA in hexane containing small amounts of methanol are shown in Figure 4. These low concentrations of ethanol result in a loss of the structured emission, which is replaced by a longer-wavelength unstructured emission. As the solvent polarity is increased further,

the emission spectra continue a more gradual shift to longer wavelengths. These spectra suggest that the emission of 2- AA is sensitive to both specific solvent effects, and general solvent effects in more polar solvents.

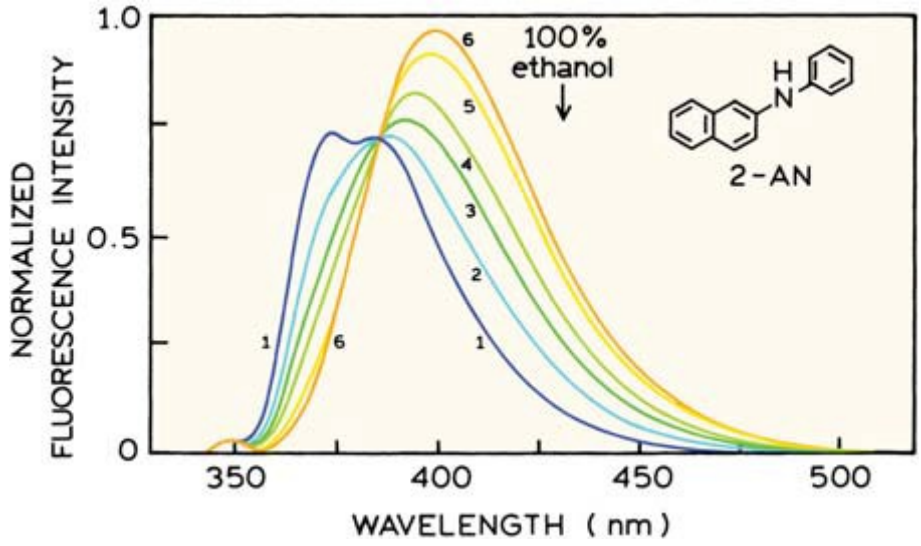

Figure 3. Fluorescence emission spectra of 2-anilinonaphthalene in cyclohexane, to which ethanol was added. These quantities were 0% (1),

0.2% (2), 0.4%, (3), 0.7% (4), 1.7% (5), and 2.7% (6). The arrow indicates the emission maximum in 100% ethanol.

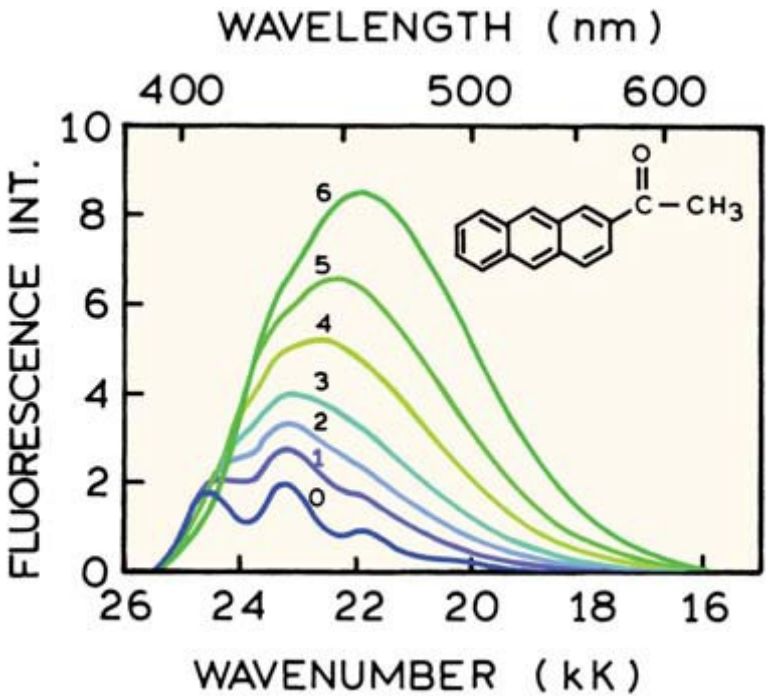

Figure 4. Fluorescence spectra of 2-acetylanthracene in methanol–hexane mixtures at 20°C. Concentrations of methanol in mol dm^{-3}: (0) 0, (1) 0.03, (2) 0.05, (3) 0.075, (4) 0.12, (5) 0.2, and (6) 0.34.

ADDITIONAL FACTORS THAT AFFECT, EMISSION SPECTRA, Locally Excited and Internal Charge-Transfer States

Depending upon solvent polarity some fluorophores can display emission before or after charge separation. One example is shown in Figure 5. The initially excited state is called the locally excited (LE) state. In low-polarity solvents FPP (9H-pyrrolo[1,2-a]indole) emits at short wavelengths from the LE state. As the solvent polarity increases a new longer wavelength emission appears. This longer-wavelength emission (lower panel) is due to an internal charge-transfer (ICT) state, which forms rapidly

following excitation. In this case the two ends of the fluorophore are held rigidly by the methylene bridge, so that formation of the ICT state does not depend on the twisting. There have been a large number of papers on conformational changes in the excited state fluorophore to form a twisted internal charge transfer (TICT) state in a variety of molecules. There seems to be a lack of agreement on the need for twisting. To avoid stating an opinion on this topic, we will simply refer to such states as ICT states. Another example of LE and ICT emission is given by Laurdan. Part of the large spectral shift displayed by Laurdan is due to emission from the locally excited state (LE), which occurs near 400 nm, as well as from an ICT state emitting at longer wavelengths. This new blue-emitting state was more easily seen in ethanol at low temperatures (Figure 6). As the temperature is decreased the emission maximum shifts from about 490 to 455 nm. As the temperature is lowered to –190 a new emission appears with a maximum near 420 nm. Although solvent relaxation usually proceeds faster at higher temperatures, high temperature can also prevent the alignment of solvent dipoles. This effect can also prevent the alignment of solvent dipoles. This effect is seen for Laurdan in ethanol at 20°C (Figure 6, right). This emission spectrum is blue-shifted relative to the emission spectrum in ethanol at –40°C. In general, the most pronounced red shifts occur at temperatures at which the solvent is fluid enough to reorient prior to fluorescence emission but thermal energy is not so great as to disrupt these orientations.

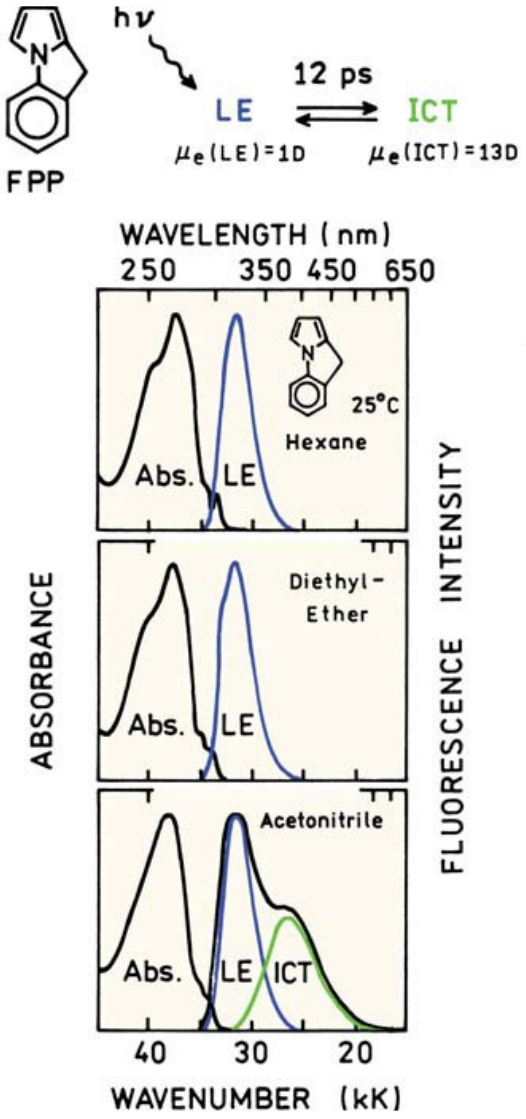

Figure 5. Emission spectra of FPP in several solvents.

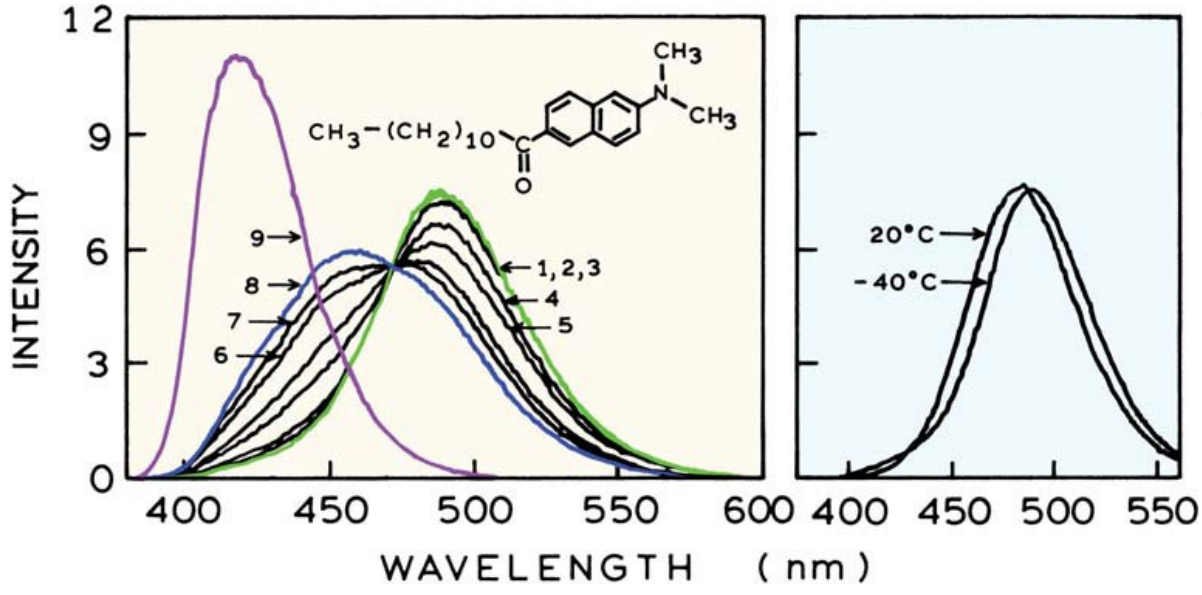

Figure 6. Emission spectra of Laurdan in ethanol at –50°C (1), –60°C (2), –70°C (3), –80°C (4), –85°C (5), –90°C (6), –100°C (7), –110°C (8), and –190°C (9). The panel on the right compares the emission spectra of Laurdan at –40 and 20°C.

The unusual temperature-dependent spectra displayed by Laurdan were explained by the presence of emission from the locally excited (LE) state and from the internal charge-transfer (ICT) state. In the LE state the excitation is localized on the naphthalene ring, so that the molecule is not very polar. In this LE state the amino and carbonyl groups are not part of the delocalized electron system. At higher temperature the ICT state forms, with complete charge transfer from the amino group to the carbonyl group. Some authors propose that twisting of the dimethyl amino group is required to allow the nitrogen electrons to be in conjugation with the naphthalene ring. Hence, the large spectral shift displayed by Prodan-like molecules (figure 7) is somewhat misleading. Part of the shift from 420 to 455 nm is due to formation of the TICT state. The remaining shift from 455 to 490 nm is due to the orientation polarizability (Δf) of the solvent. Prodan is just one example of a large number of molecules that display ICT emission.

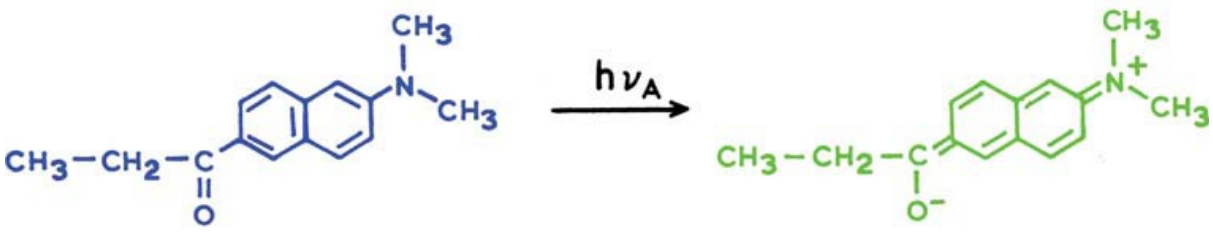

Figure 7. Charge separation in the excited state of Prodan (6-propionyl-2-(dimethylamino) naphthalene.

MECHANISMS OF QUENCHING

There are at least three mechanisms for quenching:

1. Intersystem crossing or the heavy atom effect

2. Electron exchange or Dexter interactions

3. Photoinduced electron transfer

It is often difficult to know the mechanism of quenching. The mechanisms are not mutually exclusive, and many reports indicate that quenching occurs by a combination of these mechanisms. We will use these three mechanisms as limiting cases to provide a framework of discussion.

Intersystem Crossing

Quenching by heavy atoms halogens and oxygen is thought to occur by intersystem crossing. An encounter with a heavy atom or a triplet oxygen molecule is thought to cause the excited singlet state to become an excited triplet (Figure 8). Since the triplet states are usually long lived and also quenched by oxygen, they are likely to be quenched to the ground state by the same quencher, or return to the ground state by non-radiative decay. It is not always clear which mechanism is dominant. Various reports have suggested oxygen quenching occurs by mixed mechanisms that include intersystem crossing, charge transfer, and electron exchange. Depending upon the structure of the fluorophore, quenching by halogens has also been attributed to charge transfer, intersystem crossing,

and/or electron exchange. In general it seems that halocarbons quench by intersystem crossing and halides quench by charge transfer. Additionally, many fluorophores undergo photo destruction in the presence of halocarbons.

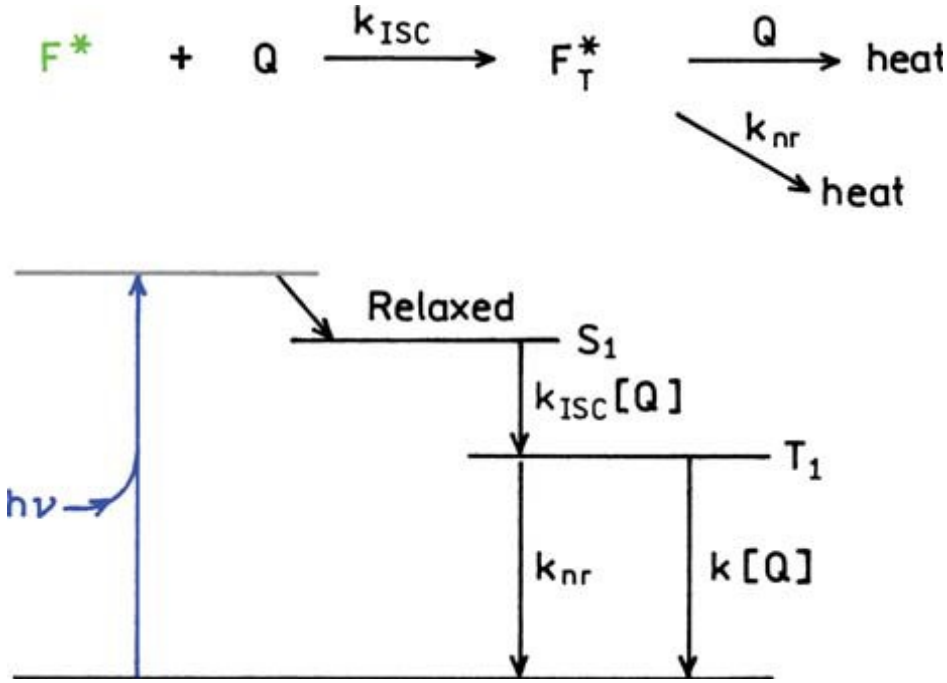

Figure 8. Quenching by intersystem crossing.