Chapter 2
Techniques

In this chapter the rudiments of the two main experimental techniques exploited in this work, i.e. fluorescence spectroscopy and neutron reflectivity, will be briefly discussed.

2.1 Fluorescence Spectroscopy

Molecular fluorescence is the emission of light from a molecule—the fluorophore—after being excited by the absorption of a photon. Fluorescence spectroscopy is a powerful technique, which provides information on the structure and dynamics of biomolecules, and for this reason it is one of the most widely used methodologies in biophysical studies. Proteins and peptides are often endowed with intrinsic fluorescence, due to the presence of Trp or Tyr residues. A great advantage of this technique is its sensitivity, which gives the possibility to work at low concentration of fluorophores.

A fluorescent molecule can be excited by light absorption from the ground state (S_0) to a higher electronic state (S_1 or S_2 in Fig. 2.1) using appropriate wavelengths (λ_1 or λ_2) [9], corresponding to the maxima of absorption. In each electronic state, the fluorophore can occupy different vibrational energy levels, but it usually rapidly relaxes to the lower vibrational level of S_1, transferring the excess of its vibrational energy to the solvent by molecular collisions. If the fluorophore is excited to the second singlet state S_2, it rapidly decays to S_1. This process is called internal conversion.

From this level, the molecule spontaneously returns to the ground state. The relaxation to the ground state is in this case a spin-allowed transition, and occurs in 10^{-5}–10^{-8} s, with the emission of a photon. If an intersystem crossing takes place, leading to a triplet excited state, the phenomenon of phosphorescence occurs.

The radiative decay is in competition with all other nonradiative relaxation pathways; a quantitative way to characterize the importance of the radiative

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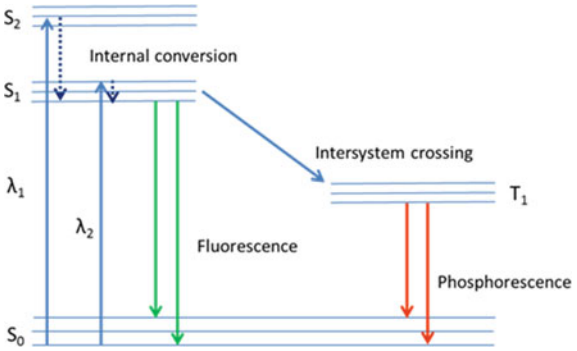

Fig. 2.1 Schematic representation of the phenomenon of fluorescence with the Jablonski diagram

process with respect to the others is the quantum yield, defined as the ratio between emitted and absorbed photons:

$$q = \frac{n_f}{n_a} = \frac{k_r}{k_r + k_{nr}}$$

in which k_r is the rate constant of radiative processes, while k_{nr} is the rate constant of nonradiative processes. Therefore, this parameter represents the probability that an excited molecule returns to the ground sate by emitting a photon. It can be close to unit if k_{nr} is much smaller than k_r [7].

2.1.1 Steady-State Fluorescence

In steady-state measurements, the sample is illuminated with a continuous light beam and the intensity of emission is recorded as a function of wavelength. The steady-state condition is reached in few ns; thus, a constant fraction of fluorophores is excited during the collection of the emission spectrum [7]. This spectrum represents the probability distribution of all the transitions from the lower vibrational state of S_1 to each vibrational state of S_0. The emission spectrum is characteristic for each fluorophore; the shape and position of spectral peaks are extremely sensitive to the molecular properties of the medium. This is due to the perturbation of fluorophore's energy levels caused by electrostatic or dipole-dipole interactions, which are stronger in polar solvents. The effects of solvent polarity are the main origin of the difference between absorption and emission wavelengths (the Stoke's shift) [1, 7].

Most of the peptides investigated in this work have one or more Trp residues in their sequence, and therefore are intrinsically fluorescent. The intensity and the position of the maximum in the emission spectrum can give information about the position of a peptide in the lipid membrane. When the peptide is inserted within the membrane, its intensity usually increases, because the more viscous nature of

the lipid bilayer reduces the rate of nonradiative decays with respect to the aqueous phase. Furthermore, the position of the maximum emission is shifted to lower wavelengths, because of the lower polarity of the membrane environment.

A measurement of the blue shift that occurs when a peptide binds to a lipid bilayer can be provided by the average wavelength of the spectrum, which is defined as [1]

$$\langle \lambda \rangle = \Sigma_i \lambda_i I_i / \Sigma_i I_i$$

where λ_i represents each wavelength of the emission spectrum and I_i is the corresponding intensity. Therefore, the variation of emission intensity and of the wavelength of maximum emission (or of the average wavelength) represents an evidence of peptide–membrane association.

2.1.2 Time-Resolved Fluorescence

Time-resolved fluorescence provides a characterization of the excited state lifetime. When a homogeneous population of fluorescent molecules is excited with a short light pulse, the decay of the emitted intensity can be described by a monoexponential law:

$$F(t) = F_0 \exp(-t/\tau)$$

where F_0 is the fluorescence intensity immediately after the pulse and τ is the fluorescence lifetime, i.e. The average time that a molecule spends in the excited state before the relaxation to the ground state. τ is related to the rate constants of radiative and nonradiative processes by the following equation:

$$\tau = \frac{1}{k_r + k_{nr}}$$

The steady-state emission intensity is related to the fluorescence lifetime by the following equation:

$$I_{ss} = \int_0^\infty F_0 e^{-t/\tau} dt = F_0 \tau$$

Time-resolved fluorescence is particularly useful to characterize heterogeneous systems in which the fluorophore exists in different states (different conformations, aggregation states, phases). In this case, each population of molecules is characterized by a different lifetime, due to the fact that their k_{nr} values are different

(while k_r is quite similar, being independent from the environment of the fluorophore). The lifetime decay is given by the contribution of all the present species:

$$F(t) = F(0) \sum_i \alpha_i e^{-t/\tau i}$$

Here the pre-exponential parameters α_i correspond to the molar fractions of the different species present in the sample.

A further advantage of lifetime measurements is that they are much less affected by experimental artifacts than steady-state experiments. For instance, they are independent on fluorophore concentration and do not suffer from inner-filter effect (apparent reduction in steady-state intensity due to absorption of the exciting or emitted light by the sample itself).

2.1.3 FRET

The phenomenon called Förster Resonance Energy Transfer (FRET) is a very useful tool to determine intermolecular or intramolecular distances. This process consists in the transfer of energy from a donor, in its excited state, to an acceptor, in its ground state [7]. It is a resonance process due to the dipolar interaction between the two fluorophores, and it does not involve the emission of photons. The necessary condition for FRET to occur is that the donor's emission spectrum overlaps, at least partially, to the acceptor's absorption. The time constant of the FRET phenomenon is described by the following equation

$$k_T = \frac{1}{\tau_D} \cdot \frac{3\kappa^2}{2} \cdot \left(\frac{R_0}{r}\right)^6$$

where τ_D is the donor's lifetime in the absence of acceptors, κ^2 is a factor describing the relative orientation of the transition dipoles of the two molecules, assumed equal to 2/3 if they are moving rapidly during the fluorescence lifetime; r is the distance between the two fluorophores; R_0 is called the Förster radius, that is, the distance occurring between donor and acceptor when the transfer efficiency is 0.5. Förster distances are typically in a range between 15 and 60 Å, which is a very interesting range, since it includes for example the typical size of proteins, or the thickness of a lipid bilayer. From an experimental point of view, the observed phenomenon is a decrease in the fluorescence intensity in the donor's spectrum, due to the decrease in its fluorescence lifetime and in its quantum yield, and an increase in that of the acceptor. Indeed, the rate constant of the excited state decay of the donor is

$$k^D = k_{nr}^D + k_r^D + k_T$$

where k_r^D and k_{nr}^D are the rate constants of radiative and of all other nonradiative processes of the unperturbed donor and k_T is the rate constant of the energy transfer process.

FRET efficiency is defined as the probability that an excited donor relaxes to the ground state by energy transfer

$$E = \frac{k_T}{k_T + k_r^D + k_{nr}^D}$$

It is strictly related to the distance between donor and acceptor molecules, as shown by the following equation

$$E = \frac{1}{1 + \left(\frac{3}{2}k^2\right)^{-1}\left(\frac{r}{R_0}\right)^6}$$

This result indicates that FRET can be exploited to investigate the relative position of two fluorophores in a biological environment, and also to explore interactions occurring in the range of several Ångstroms. The range of distances in which FRET can be detected is comprised approximately between $R_0/2$ and $2R_0$.

In our case, FRET experiments are suitable to establish the interaction of a peptide with the lipid bilayer, exploiting the energy transfer occurring between the peptide Trp residues and a membrane-bound acceptor [1].

2.1.4 Fluorescence Anisotropy

Fluorescence anisotropy is used to determine the rotational dynamics of a fluorophore. Since molecular motions are significantly slower inside a lipid bilayer than in water, this technique can be used also to assess peptide-membrane association.

In a fluorescence anisotropy experiment the sample is excited with polarized light. The molecules in the sample are randomly oriented before the excitation, but the probability for a molecule to be excited is dependent on the factor $\cos^2\theta$, where θ is the angle between the directions of the polarization of the exciting light and of the transition dipole of the fluorophore. Thus, the molecules having the transition dipole oriented in the same direction of the exciting light have the highest probability of excitation. This phenomenon is called photoselection. The light emitted from the photoselected fluorophores is also polarized preferentially along the direction of the emission transition dipole, again according to a $\cos^2\theta$ law. By measuring the degree of polarization of the emitted light it is therefore possible to assess the degree of molecular motions that take place during the fluorophore's excited state lifetime.

If the molecular motions are very fast with respect to τ, the preferential orientation is lost during the lifetime decay, and the emitted light is unpolarized, due to the random orientation of the transition dipoles. By contrast, if the motions are slow, the emitted light is anisotropic (i.e. exhibits a preferential polarization). Thus, anisotropy measurements can provide information about the rotational motions of a fluorophore in the nanoseconds time scale, and about the viscosity of its environment.

Experimentally, the anisotropy is measured as

$$r(t) = \frac{I_{vv}(t) - GI_{vh}(t)}{I_{vv}(t) + 2GI_{vh}(t)}$$

where the subscripted indexes indicate the vertical (v) or horizontal (h) orientation of excitation and emission polarizers with respect to the instrumental plane, and G is an instrumental parameter, defined as

$$G = I_{hv}/I_{hh}$$

2.2 Neutron Reflectivity

Neutron spectroscopy techniques are a powerful tool for the study and characterization of biological systems and soft matter, like lipid membranes, rich in light atoms C, H, N and O, whose nuclei are good neutron scatterers. [3, 4, 6].

Since neutrons interact with atomic nuclei only weakly, they can penetrate deeply within samples, and provide information about complex structures and buried interfaces [3].

Neutron reflectivity allows the characterization of the composition and of the irregularities of biological materials, in a length scale between 10 and 5000 Å.

In the specular neutron reflectivity technique, the reflected intensity is measured as a function of the incident angle [2]. Experiments are performed at a grazing angle, usually varying from 0.01 to 5° [3]; sample and detector are moved in order to keep the incident and reflected angles equal.

Specular reflectivity is defined as the ratio between the reflected and the incoming beam intensities, and it is measured as a function of the scattering vector, Q, perpendicular to the surface plane:

$$R = \frac{16\pi^2}{Q^2}\left|\widetilde{\rho}(Q)^2\right|$$

where Q, is defined as

$$Q = \frac{4\pi}{\lambda} sen\,\theta$$

and $\widetilde{\rho}(Q)$ is the Fourier transform of $\rho(z)$, the scattering length density (SLD), that is the parameter providing informations about the structure along the normal axis to the surface. Thus, there is a direct relationship between reflectivity and structure. The SLD is formally defined as $\rho(z) = \Sigma_i \nu_i b_i$, where ν_i is the number of nuclei of type i per volume unit and b_i is the neutron scattering length of those nuclei. During the experiments, a profile of the reflectivity R as a function of Q is acquired.

An important characteristic of neutron spectroscopy is its extreme and unique sensitivity to the substitution of hydrogen by deuterium, due to the fact that neutrons are scattered very differently from these two nuclei. Indeed, the scattering lengths values are –0.37 for hydrogen and 0.67 for deuterium [5]. Thus, it is possible to perform experiments in different H_2O/D_2O mixtures, to enhance the signal of some atoms and minimize that of other ones. This technique is known as the *contrast variation method*; all the profiles obtained in different media are simultaneously fitted. This method is used to have enough constrains to exactly define the physically meaningful model.

The data fitting procedure is based on the Parratt's recursion relation [8], and it often involves the construction of a model of the interface that can be represented by a series of parallel layers of homogeneous material. In the present case, the lipid bilayer has been divided into three sublayers, i.e. the outer headgroups region, the tails region, and the inner headgroups region.

References

1. Bocchinfuso G, Bobone S Palleschi A, Stella L (2011) Fluorescence spectroscopy and molecular dynamics simulations in studies on the mechanism of membrane destabilization by antimicrobial peptides. Cell Mol Life Sci 68:2281–2301
2. Dabowska AP, Fragneto G, Hughes AV, Quinn PJ, Lawrence MJ (2009) Specular neutron reflectivity studies on the interaction of Cytochrome c with supported phosphatidylcholine bilayers doped with phosphotidylserine. Langmuir 25:4203–4210
3. Fragneto, G. and Rheinstaedter (2007) Structural and dynamical studies from bio-mimetic systems: an overview. C R Phys 8:865–883
4. Fragneto G, Graner F, Charitat T, Dubos P, Bellet-Amalric EB (2000) Interaction of the third helix of antennapedia homeodomain with a deposited phospholipid bilayer: a neutron reflectivity structural study. Langmuir 16:4581–4588
5. Jacrot B (1976) The study of biological structures by neutron scattering from solution. Rep Prog Phys 39:911
6. Krueger S (2001) Neutron reflection from interfaces with biological and biomimetic materials. Curr Op in Coll Interface Sci 6:111–117
7. Lakowicz JR (2006) Principles of Fluorescence Spectroscopy, 3rd edn. Springer, berlin
8. Parrat LG (1954) Surface studies of solids by total reflection of X-rays. Phys Rev 95:359–369
9. Skoog DA, Leary JJ (1992) Principles of instrumental analysis. Saunders College Pub, New York

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