

# From Lipid Phases to Membrane Protein Organization: Fluorescence Methodologies in the Study of Lipid-Protein Interactions

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## 1.1 General Background

The fluid mosaic model of biological membranes (Singer and Nicolson 1972) emphasizes membrane fluidity and free lateral diffusion of membrane components. This has led to the generalized idea of biomembranes as solutions of proteins embedded in bilayers of randomly distributed phospholipids. The current view is moving towards the probable equal importance of lipids and proteins in the determination of the constitution, structure and dynamics of membrane domains and the hierarchic structure of the membrane, in which small patches revealed by non-random patterns of co-distribution of specific proteins are the building blocks of large aggregates (Vereb et al. 2003).

The use of model systems of membranes prepared with pure lipids and lipid mixtures in the absence or presence of peptide/proteins has proved useful in elucidating the organization, topology, and orientation of membrane proteins. To approach this study the following possibilities have to be considered: (1) diverse phase behaviour of the lipid or the lipid mixture in the absence of peptide/protein (knowledge of the phase diagram); the balance between different molecular interactions will change markedly, e.g. if the lipid is in the gel or in the fluid phase; if there is phase coexistence the protein can partition preferentially to one phase and have different aggregation states in each phase, etc.; (2) the influence of the protein on the phase behaviour (shifts in the phase diagram, creation of new regions/types of phase separation); and (3) the influence of the peptide/protein on the phase separation topology, without changing the lipid phase diagram. In fact, the formation of lipid domains is thought to be a key process in several biological functions (e.g. Simons and Ikonen 1997) and the clarification of the relationship between lipid domains and the binding and functional properties of membrane-associated proteins is an emerging area in membrane research (Hurley and Meyer 2001). The phase behaviour for a ternary lipid mixture (e.g. mimicking raft-containing membranes) can be quite complex, as well as the variation of domain size with lipid composition (de Almeida et al. 2005), and it is necessary to characterize it before studying the effect of the protein. Other kinds of heterogeneities, such as the lipid annulus around a transmembrane protein, which do not correspond to a true phase separation, may also occur. Depending on the proportion of peptide/protein and lipid, or the type of lipid, the protein/peptide system can undergo strong alterations, such as peptide displacement from a position parallel to a perpendicular one, in relation to the membrane axis. Some important prin-

ciples have been established, namely the prediction of membrane protein topology (Jayasinghe et al. 2001), the hydrophobic mismatch principle (Mouritsen and Bloom 1984), and also more specific aspects like the distinct roles of tryptophan and lysine anchors (de Planque et al. 1999). Approaches based on fluorescence spectroscopic techniques, due to their intrinsic sensitivity, suitable time-scale, non-invasive nature and minimum perturbation, can be useful in studying all these aspects of lipid-protein interaction. Examples of when and how to use this technique will be given in the later sections of this chapter, after a brief review of the most important fluorescence methodologies and parameters, and a survey of the more useful fluorophores in these types of studies. Direct inspection of fluorescence steady-state data allows us to qualitatively assess the physical details of the system. On the other hand, state-of-the-art analysis of time-resolved fluorescence data (e.g. FRET and anisotropy) can give detailed information on the dynamics as well as the topology (e.g. vesicle interaction and phospholipid lateral distribution).

## 1.2 Fluorescence Methodologies

### 1.2.1 Fluorescence Resonance Energy Transfer

Fluorescence resonance energy transfer (FRET) is a photophysical phenomenon upon which the excited state of a molecule, the donor, is transferred to another, the acceptor. This latter molecule can be either identical to the donor, or a different species, resulting in homotransfer (energy migration, homo-FRET) or heterotransfer (hetero-FRET). As a result of FRET, the donor fluorescence is quenched, and the acceptor becomes excited (and may fluoresce). The rate constant for FRET,  $k_T$ , depends on the inverse of the sixth power of the separation distance between the donor and acceptor,  $R$  (Förster 1949). The FRET efficiency,  $E$ , can be obtained experimentally from the reduction of fluorescence intensity ( $I_{DA}$ ) or lifetime ( $\tau_{DA}$ ) in the presence of the acceptor, relative to their values in the absence of the acceptor ( $I_D$  and  $\tau_D$ , respectively):

$$E = 1 - \frac{I_{DA}}{I_D} = 1 - \frac{\tau_{DA}}{\tau_D} \quad (1.1)$$

In the case that the donor decay is described by a sum of exponentials (where  $\alpha_i$  are the normalized pre-exponential factors):

$$i(t) = \sum_{i=1}^n \alpha_i \exp(-t / \tau_i) \quad (1.2)$$

$\tau_{DA}$  and  $\tau_D$  in Eq. 1.1 should be replaced by the lifetime quantum yields  $\langle \tau \rangle_{DA}$  and  $\langle \tau \rangle_D$ , defined by (e.g. Lakowicz 1999)

$$\langle \tau \rangle = \sum_{i=1}^n \alpha_i \tau_i \quad (1.3)$$

In membranes, each donor is surrounded by a distribution of acceptors, at varying distances, therefore, FRET kinetics are considerably more complex. For the derivation of the donor decay law in this situation, the starting point is usually the master equation

$$i_{DA}(t) = \exp(-t/\tau_D) \prod_{i=1}^{N_A} \exp\left[-\left(\frac{t}{\tau_D}\right)\left(\frac{R_0}{R_i}\right)^6\right] \quad (1.4)$$

where  $N_A$  is the number of acceptors, and  $R_i$  is the distance between the donor molecule in question and the  $i$ th acceptor molecule. Although this equation concerns a single donor molecule, it can be used for any situation where all donor molecules are equivalent. In this way, expressions for the donor decay for geometries relevant to bilayer systems have been obtained. These include FRET to an ensemble of acceptors in an infinite plane, from donors located either in the same plane (planar geometry or *cis* FRET) or 2D FRET, as in Eq. 1.5 (Tweet et al. 1964), or in another parallel plane (bilayer geometry or *trans* FRET), as in Eq. 1.6 (Davenport et al. 1985).

$$i_{DA, cis}(t) = \exp\left(-\frac{t}{\tau_D}\right) \exp\left\{-\pi R_0^2 n \gamma\left[\frac{2}{3}\left(\frac{R_0}{R_e}\right)^6\left(\frac{t}{\tau_D}\right)\right]\left(\frac{t}{\tau_D}\right)^{1/3}\right\} \\ \cdot \exp\left\{\pi R_e^2 n \left(1 - \exp\left[-\left(\frac{R_0}{R_e}\right)^6\left(\frac{t}{\tau_D}\right)\right]\right)\right\} \quad (1.5)$$

$$i_{DA, trans}(t) = \exp\left(-\frac{t}{\tau_D}\right) \exp\left\{-2n\pi l^2 \int_0^{\frac{1}{\sqrt{l^2+R_e^2}}} \frac{1 - \exp(-tb^3\alpha^6)}{\alpha^6} d\alpha\right\} \quad (1.6)$$

In these equations,  $n$  is the number of acceptors per unit area,  $\gamma$  is the incomplete gamma function (see Lakowicz 1999, page 426),  $R_e$  is the distance of closest approach between donor and acceptor molecules,  $l$  is the distance between the planes of donors and acceptors and  $b = (R_0/l)^2\tau_D^{-1/3}$ . In the case that  $R_e \ll R_0$  (in practice, if  $R_e < R_0/4$ ), the incomplete gamma term in Eq. 1.5 can be replaced by  $\Gamma(2/3)$  (where  $\Gamma$  is now the complete gamma function) and the last exponential term in that equation can be omitted, whereas the upper limit in the integral in Eq. 1.6 becomes 1.

In the above cases, a uniform distribution of acceptors was always assumed (for the kinetics of FRET in the case of separation of two infinite phases with a uniform acceptor distribution within each phase see, e.g. Loura et al. 2001). A relevant situation in the context of lipid-protein interaction for which this clearly does not hold is when the composition of the annular lipid region surrounding the protein is different from that of the bulk. In a recent study (Fernandes et al. 2004), FRET from a protein-located donor (in the centre of the bilayer) to a labelled-phospholipid acceptor, with fluorophores located on both lipid/water interfaces, was modelled assuming a single layer of annular lipid. The model assumes two populations of energy transfer acceptors, one located in the single annular lipid

shell around the protein and the other outside the shell. The donor fluorescence decay curve has FRET contributions from both populations:

$$i_{DA}(t) = i_D(t) \rho_{\text{annular}}(t) \rho_{\text{random}}(t) \quad (1.7)$$

Here  $i_D$  and  $i_{DA}$  are the donor fluorescence decay in the absence and presence of acceptors, respectively, and  $\rho_{\text{annular}}$  and  $\rho_{\text{random}}$  are the FRET contributions arising from energy transfer to annular labelled lipids and to uniformly distributed labelled lipids outside the annular shell, respectively. Considering a hexagonal-type geometry for the protein–lipid arrangement, each donor protein will be surrounded by 12 annular lipids. In bilayers composed by both labelled and unlabelled phospholipids, these 12 sites will be available for both of them. The probability  $\mu$  of one of these sites being occupied by a labelled phospholipid is given by

$$\mu = K_S \cdot n_{\text{acceptor}} / (n_{\text{acceptor}} + n_{\text{lipid}}) \quad (1.8)$$

Here,  $n_{\text{acceptor}}$  is the concentration of labelled lipid, and  $n_{\text{lipid}}$  is the concentration of unlabelled lipid.  $K_S$  is the relative association constant, which reports the relative affinity of the labelled and unlabelled phospholipids. Using a binomial distribution, the probability of each occupation number (0–12 sites occupied simultaneously by labelled lipid), and finally the FRET contribution arising from energy transfer to annular lipids is computed

$$\rho_{\text{annular}}(t) = \sum_{n=0}^{12} \exp(-nk_T t) \binom{12}{n} \mu^n (1-\mu)^{12-n} \quad (1.9)$$

The FRET contribution from acceptors uniformly distributed outside the annular region in two different planes at the same distance to the donor plane (from the centre of the bilayer to both leaflets) is given by the latter term of Eq. 1.6, in which  $n$  must be corrected for the presence of labelled lipid molecules in the annular region, which therefore are not part of the uniformly distributed acceptor pool.

In all situations, the theoretical energy transfer efficiency  $E$  is readily calculated by numerical integration,

$$E = 1 - \frac{\int_0^{\infty} i_{DA}(t) dt}{\int_0^{\infty} i_D(t) dt} \quad (1.10)$$

and can be compared with the experimental observable obtained from Eq. 1.1.

Despite having the obvious advantage of only requiring a single fluorophore, the use of homotransfer is more restricted than that of heterotransfer. One reason for this is that homotransfer does not lead to a reduction in donor fluorescence intensity or lifetime, because the donor excited state population is not dimin-

ished during the act of transfer. In practice, the sole observable which reflects the phenomenon is a reduction in fluorescence anisotropy (see Sect. 1.2.2), the measurement of which requires polarizers and, because these lead to a considerable reduction in the detected emission, often a larger amount of fluorophore (relative to that which would be used in an intensity measurement) is needed for a given precision. In the case that instrumentation is not a problem, the decrease in anisotropy is quite clear.

More importantly, the theory of depolarization due to homotransfer is more complicated than that of heterotransfer, because (1) there is the possibility of back-transfer to the directly excited donor, or transfer to any donor, eventually involving a large number of transfer steps, and (2) since fluorescence anisotropy is the relevant observable, in addition to FRET, another source of depolarization is fluorophore rotation. If rotation and FRET occur in the same time-scale, the two phenomena are coupled. This is why most theories for homotransfer assume static dipoles (see Kowski 1983 and Van der Meer et al. 1994 for reviews and discussion of the theory), even the user-friendly numerical simulations of Snyder and Freire (1982). Some authors assume that the experimental anisotropy decay is the product of a rotational depolarization term by a FRET depolarization term obtained from a static-dipole theory (e.g. Medhage et al. 1992), and therefore the FRET term can be recovered, e.g. from the faster component in the anisotropy decay (Sharma et al. 2004). It must be understood that this independence of rotation and transfer constitutes an approximation (unless the time-scales for the two phenomena are very distinct), whose severity is not easy to ascertain. The coupling of rotation and FRET in the measured anisotropy is therefore the main obstacle to quantitative data analysis of homotransfer. However, this has not completely stopped the use of the latter in the context of lipid-protein interaction, and examples will be given in Sect. 1.4.

### 1.2.2 Anisotropy

Molecular electronic transitions are characterized by absorption and emission dipoles (and their associated transition moments) fixed with respect to the molecular frame. When an assembly of randomly oriented molecules is illuminated with polarized light of suitable frequency the probability of absorption is proportional to  $\cos^2\gamma$ , where  $\gamma$  represents the angle between the absorption transition moment and the exciting electric vector. This anisotropy in the photoabsorption process causes preferential excitation (photoselection) of those molecules oriented forming angles  $\gamma$  close to zero. The orientational anisotropy is a maximum at the instant of excitation ( $t = 0$ ) and will decrease as a function of time due, among other things, to the Brownian motion experienced by the excited molecules. Since these random motions depend on the shape and size of the molecules and on the viscosity and temperature of the solvent, the decrease of the orientational anisotropy will be related to these parameters. Therefore, any method able to detect the initial orientation of the photoselected population and monitor its temporal evolution will give important information about the dynamic properties of the

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