Interaction of peptides with binary phospholipid membranes: application of fluorescence methodologies

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\textbf{Abstract}

The application of fluorescence methodologies to obtain information about the extent, dynamics and topology of peptide interaction with binary phospholipid (mainly zwitterionic/anionic) mixtures is reviewed. First, general approaches based on peptide (tryptophan residues) fluorescence properties that give information about its partition, location and dynamics will be presented. Then, methodologies based on membrane probes fluorescence that report the influence of peptide binding and/or incorporation on the lateral organization (phase separation) of membrane phospholipids will be described. Specific examples taken from the literature that illustrate both situations are presented as well as formalisms for data analysis. It is shown that steady-state and time-resolved fluorescence data (particularly important in the case of fluorescence resonance energy transfer studies) give complementary information, allowing a molecular picture of peptide interaction with biphasic systems to be drawn.

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1. Introduction

The interaction between peptides and lipid bilayers has been a growing area of study in the last years. Knowledge about the molecular mechanism of polypeptide interaction with, insertion into and final organization within membranes is critical for understanding the activity of many hormones, host-defense peptides and lipopeptides, as well as toxins (\textit{White et al., 1998; Epand and Vogel, 1999}). Additionally, the use of several carefully chosen synthetic or naturally occurring peptide segments from integral membrane proteins or from membrane binding proteins has helped to clarify the principles that govern molecular recognition between membrane-spanning polypeptides and binding/association at the membrane interface, respectively (\textit{Deber et al., 1999; Shai, 2001}).

The study of the interaction of model peptides with a zwitterionic lipid bilayer was fundamental.
to establish the hydrophilic/hydrophobic ener-
getics of membrane proteins insertion (Wimley
and White, 1996). Nonetheless, the importance of
the complex composition of natural membranes
has long been realized, and the presence of a single
additional component, e.g., the use of a binary
zwitterionic/anionic phospholipid lipid mixture, is
sufficient to allow for a panoply of new interac-
tions to be established. The following possibilities
have to be considered: (i) phase behavior of the
binary lipid mixture in the absence of peptide
(knowledge of the temperature/composition phase
diagram); (ii) influence of the peptide on the phase
behavior (shifts in the phase diagram, creation of
new regions/types of phase separation); and (iii)
influence of the peptide on the peptide on the phase
separation topology. In fact, the formation of lipid
domains is thought to be a key process in several
biological functions (Welti and Glaser, 1994; Simons
and Ikonen, 1997) and the clarification of the relation-
ship between lipid domains and the binding and
functional properties of membrane-associated pro-
teins is an emerging area in membrane research
(Johnson and Cornell, 1999; Hurley and Meyer,
2001). It should be stressed that acidic phospho-
lipids located in the inner leaflet of the plasma
membrane are particularly important in this re-
gard, because several membrane-active peptides
and proteins, e.g. associated with signal transduc-
tion, have a cluster of basic residues that target
them to the lipid bilayer through electrostatic
interactions (Buckland and Wilton, 2000).

In all the above issues, fluorescence spectro-
scopy has assumed an important role as a struc-
tural tool providing information about the
polymerization and dynamics of polypeptides. This
work deals with the interaction of peptides with
binary phospholipid mixtures (mainly zwitterionic/
anionic) and is centered on the application of
fluorescence spectroscopy techniques. Instead of
being an extensive review, it is focused on selected
examples from the literature (and from our own
work) that will be described to illustrate each
methodology. Out of the scope of this review are
e.g., fluorescence microscopy techniques and the-
oretical rationalization of peptide–lipid mixture
interactions. Instead, emphasis is given to the
experimental evidence that can be directly ob-
tained from different types of fluorescence spectro-
scopy experiments. For practical reasons, the
paper is divided into two main sections: methodol-
ogies based on peptide intrinsic fluorescence
properties (reporting the degree of interaction
with the bilayer/immediate environment of the
peptide) and methodologies based on membrane
probe fluorescence (reporting what happens to the
membrane upon peptide binding).

2. The peptide point of view: techniques based
on the emission of Trp residues

Application of fluorescence spectroscopy to the
study of membrane-interacting peptides has
usually taken advantage of the presence of natu-
ally occurring or engineered tryptophan (Trp)
residues in their primary structure. Several proper-
tries of these fluorescent intrinsic probes of the
peptides, including fluorescence lifetime and quan-
tum yield, are environmentally dependent, and can
be used to determine its membrane/water partition
coefficient. In addition, spectral shifts undergone
by the emission spectra of Trp residues, as well as
changes in accessibility to aqueous quenchers can
be exploited to characterize peptide membrane
binding. Further information about the transverse
location (degree of penetration) of the membrane-
bound peptide molecules can be gained through in
depth-dependent fluorescence quenching experi-
ments.

2.1. Peptide lipid/water partition coefficient

The association of polypeptides to membranes is
best described quantitatively in terms of a parti-
tion equilibrium between the aqueous and the lipid
bilayer phase for amphipathic polypeptides, P (for
a review see White et al., 1998).

\[
K_p = \frac{n_L^p}{n_W^p} \frac{n_W^p}{n_L^p} \frac{n_L}{n_W}
\]  

(1)

where \(K_p\) represents the mole-fraction partition
coefficient of the peptide, \(n_L\) and \(n_W\) are the
amount (in mole) of water and lipid in each sample, and $n_p^W$ is the amount of peptide molecules present in each phase ($i = W$, aqueous phase; $i = L$, lipid phase, respectively). In all partitioning experiments, it is reasonable to assume that $n_p^W \gg n_p^L$ and, because high membrane-bound concentrations of the peptide are usually avoided to prevent deviations from ideal partitioning due to peptide–peptide interactions at the water–membrane surface or in the lipid bilayer, it can also be considered that $n_L \gg n_L^W$. In these circumstances, Eq. (1) can be simplified:

$$K_p^i = \frac{n_p^i}{n_p^W} \quad (2)$$

From Eq. (2), and considering that $[P]^i = [P]^W + [P]^L$, the membrane-bound peptide mole-

fraction, $x_L$, is derived as a function of the phospholipid concentration used $[L]$:

$$x_L = \frac{K_p^L [L]}{[W] + K_p^L} \quad (3)$$

where $[W]$ is the molar concentration of water.

The changes undergone by the peptides’ fluorescence properties can be used to distinguish between free and membrane-bound peptide populations, either by steady-state (fluorescence intensity) or time-resolved fluorescence measurements (fluorescence decays), and therefore can be applied to quantitatively evaluate its interaction with the lipid bilayers.

The fluorescence intensity decay of single Trp-containing peptides usually does not follow simple exponential kinetics, and the complex decays obtained may be empirically described by a number (up to 3 or 4) of exponential components,

$$I(t) = \sum_{i=1}^{n} x_i e^{-t/\tau_i} \quad (4)$$

where $x_i$ and $\tau_i$ are the normalized amplitude ($\Sigma x_i = 1$) and lifetime of the $i$th decay component, respectively. From the fluorescence intensity decay kinetics of Trp residues, its amplitude-weighted mean fluorescence lifetime, $\bar{\tau}$, can be computed by

$$\bar{\tau} = \sum_{i=1}^{n} x_i \tau_i \quad (5)$$

$\bar{\tau}$ is also called lifetime-weighted quantum yield because it is directly proportional to the area under the decay curve (Lakowicz, 1999).

Assuming that the molar absorption coefficient of the peptide is independent of its environment, both the steady-state fluorescence intensity, $I$, and lifetime-weighted quantum yield, $\bar{\tau}$, of the peptide can be expressed as a linear combination of their limiting values characteristic of free and membrane-bound states, $I_i$ and $\bar{\tau}_i$, respectively, weighted by the fractional population of each species, $x_i$, in a sample ($i = W$, aqueous phase; $i = L$, lipid phase):

$$I = \Sigma x_i I_i \quad (6)$$

and

$$\bar{\tau} = \Sigma x_i \bar{\tau}_i \quad (7)$$

From the previous relationships, Eqs. (8) and (9) can be derived:

$$I = I_w + \frac{(I_L - I_w)K_p^W[L]}{[W] + K_p^W[L]} \quad (8)$$

and

$$\bar{\tau} = \bar{\tau}_w + \frac{(\bar{\tau}_L - \bar{\tau}_w)K_p^W[L]}{[W] + K_p^W[L]} \quad (9)$$

By fitting the above Eq. (8) or Eq. (9) to the adequate experimental data ($I$ vs. $[L]$ or $\bar{\tau}$ vs. $[L]$), the partition coefficient of the peptide can be obtained. When the peptide does not translocate across the lipid bilayer, the overall lipid concentration should be corrected for the lipid fraction accessible for the initial partitioning of the peptide, i.e. present in the outer leaflet of the lipid bilayer.

Some authors found that quantitation of partition from fluorescence intensity changes at one particular wavelength is susceptible to error (Polozov et al., 1998). This can be circumvented if the whole peptide emission spectra obtained during its titration with lipid are used in the fitting procedure described above (Eq. (8)) instead of its fluorescence intensity measured at a single wavelength $\lambda$. It is also very common the use of a binding
formalism to discrete binding sites (e.g., De Kroon et al., 1990). In this case there are two parameters to be recovered, the dissociation constant $K_d$ and the number $n$ of lipid molecules that constitute one binding site. If $n = 1$, this formalism is equivalent to that described above (e.g., Ding et al., 2001). If this is not the case, and one wants to compare data from different works, the best is to apply the equations presented here to the raw data on which this second formalism was applied.

The phase separation in biphasic systems can modulate the extent of interaction of a peptide with a membrane, as evaluated from its partition coefficient. For the peptide $\alpha$-melanocyte stimulating hormone ($\alpha$-MSH), its $K_p$ values were determined for large unilamellar vesicles (LUVs) composed of 3:1 mixtures of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC)/1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) and DMPC/1,2-dimyristoyl-sn-glycero-3-phosphoric acid (DMPA), at 20 and 37 °C, using the variation of its $\bar{\tau}$ with the lipid concentration in solution (Eq. (9)) (Contreras et al., 2001). In that work, the partition coefficients $K_p$ were defined similarly to Eq. (2), with lipid and water amounts ($n_L$ and $n_W$, respectively) replaced by their respective volumes, leading to:

$$K_p' = K_p \frac{\gamma_w}{\gamma_L}$$  \hspace{1cm} (10)$$

where $\gamma_i$ is the molar volume of water, $i = W$ or lipid, $i = L$. The fitting of Eq. (9) to the experimental data is depicted in Fig. 1, and the recovered best-fit parameters (partition coefficients, $K_p'$, and the peptide lifetime-weighted quantum yield in the membrane phase, $\bar{\tau}_L$) are shown in Table 1.

At the lower temperature (20 °C) both systems are in the gel phase, and for the DMPC/DMPA mixture the existence of phase separation can be concluded from its complex DSC thermogram (Graham et al., 1985). At variance, DMPC and DMPG are completely miscible both below and above the transition temperature, and a single peak is observed in its DSC thermogram. The main driving force for the lipid–peptide interaction is the electrostatic interaction of the positive peptide (+1) with the lipid mixtures, prepared in both cases with the same amount of negatively charged lipid (25%). In this way, it is not surprising that all the partition coefficients obtained have the same order of magnitude (Table 1). However, variations are observed which can be ascribed to the pre-existence of phase domains. For the homogeneous DMPC/DMPG system, the partition coefficient is higher for the fluid as compared to the gel phase, which is the usual result in one-component vesicles (Ito et al., 1993). Surprisingly, for DMPC/DMPA the opposite behavior is verified. Although $K_p'$ for the fluid phase is similar to the one obtained with the DMPC/DMPG mixture, a higher value is obtained for the gel phase. In this case, it is proposed that the domains enriched in the anionic phospholipids induced a stronger binding of the peptide. Another possibility is that the eventual preferential interaction of the peptide with the interfaces between the domains of coexisting phases, which are expected to have a more perturbed structure, could cause an increase in its partition coefficient.

The fluorescence intensity from Trp residues generally exhibits a significant increase upon establishing an interaction with lipid bilayers. This enhancement reflects an increased quantum
yield, $\Phi$, of the Trp residues upon inclusion in a less polar environment, which is usually accompanied by a corresponding variation in its mean fluorescence excited state lifetime. However, the reverse situation has also been reported. In fact, there is no clear correlation between quantum yield and the wavelength of maximum emission in proteins with a single Trp residue (Lakowicz, 1999). Christiaens et al. (2002) have found an unusual decrease in fluorescence intensity and mean Trp lifetime for wild-type penetratin peptide and two of its analogues upon their binding to negatively charged phosphatidylserine (PS) lipid vesicles. The authors ascribed these atypical changes to peptide conformational alterations accompanying its binding to the lipid vesicles, that might reduce the distance between one or more quencher groups and the Trp residues, and therefore causing a decrease in its fluorescence intensity. These effects can be caused by several polar protein groups, which are able to quench Trp fluorescence to some extent (Chen and Barkley, 1998), such as charged lysine and arginine side chains. Another possibility is that the phospholipid headgroups themselves may quench Trp fluorescence.

### 2.2. Fluorescence spectra

In addition to the fluorescence quantum yield and lifetime, the position of the maximum of the fluorescence spectrum of Trp residues, $\lambda_{\text{max}}$, is also sensitive to its environment, and the transfer of the peptide from the aqueous (polar) environment to the lipid bilayer (less polar), characteristically causes a blue-shift of emission from around 350 nm to as low as 325 nm. This property can therefore be used to obtain a coarse description of the location/environment of peptide’s Trp residues in the membrane. For example, Liu and Deber (1997) monitored the topology of several peptides in anionic and neutral lipid micelles, from their Trp residues spectral shifts. To unravel the interplay of hydrophobicity and electrostatics in the binding of peptides into membranes, the authors studied several peptides with the general sequence K-K-A-A-A-X-A-A-A-A-A-X-A-A-W-A-A-X-A-A-K-K-K-amide (X = I, L, G or S, A2XA2). Liu and Deber found a blue-shift from 350 to approximately 340 nm in anionic micelles for all the peptides, which suggested that the peptides inserted into the micelles. However, in neutral micelles, a blue-shift of the Trp emission maxima was observed for the hydrophobic peptides A2IA2 and A2LA2, but not for the less hydrophobic peptides A2SA2 and A2GA2. The Trp residues of the latter peptides were capable of inserting into the micelles efficiently only when 20 mol% of an anionic lyso-phospholipid was included on the micelles.

In case that the spectral shift is used to study peptide–lipid interaction, the analysis of the experimental data should take into consideration the contribution to fluorescence intensity of Trp residues present in the aqueous phase. In fact, the fluorescence of a sample comes both from the fraction of peptide in the aqueous phase and from that incorporated in the membrane, and this can...
be significant even for high lipid concentrations when the peptide’s partition coefficient is low. In case that \( K_p \) is known, the molar fraction of peptide in water \( x_W \) is obtained as the difference to unity from the one in the lipid, \( x_L \) (Eq. (3)). The spectrum of the peptide in the membrane is then obtained from (Contreras et al., 2001),

\[
F^L_{\lambda} = C \left( F^{L\cdot W}_{\lambda} - x_W \frac{1}{1 + \tau_L/\tau_W} F^{W}_{\lambda} \right)
\]  

(11)

where \( F^L_{\lambda} \) is the spectral distribution (unitary area) function of the peptide experimentally determined in water \( (i = W) \), and at a certain concentration of lipid \( (i = L+W) \). \( C \) is a normalization constant.

This methodology was applied to the study of the peptide hormone \( \alpha \)-MSH, in interaction with DMPC/DMPG and DMPC/DMPA (both 3:1 mixtures) vesicles, at 20 °C (gel phase) and 37 °C (fluid phase) (Contreras et al., 2001). Emission spectra are depicted in Fig. 2, and the spectral blue-shifts obtained upon interaction with the membranes are shown in Table 1. Clearly, in the DMPC/DMPG mixture, the hydrophobicity of the environment around the Trp residue of \( \alpha \)-MSH is larger in fluid as compared to gel phase. Because the hydration of the lipid bilayers is larger in fluid crystalline phase, i.e., information on transverse location is obtained. For the DMPC/DMPA system, the opposite situation happens: a larger shift is observed for the gel phase, and this stronger interaction is probably due to the existence of phase separation, as previously described. In agreement, the values obtained for the two lipid systems in the fluid phase are identical.

In the particular case of phospholipase \( A_2 \) (PLA2) of \( Agkistrodon piscivorus piscivorus \) the fluorescence of its Trp residues can be used in a simple manner to follow the enzyme activity. PLA2 is a small water-soluble and lipid bilayer-active enzyme that catalyses the hydrolysis of phospholipids into lysophospholipids and fatty acids. In general, the PLA2 hydrolysis profile towards vesicular substrates is characterized by a period of slow hydrolysis described by a lag time that is highly dependent on the lateral organization of the lipid substrate; a reaction burst at which the hydrolysis rate increases by several orders of magnitude follows the lag time (Apitz-Castro et al., 1982). Activity measurements can therefore be used to probe lipid bilayer micro-heterogeneity (Hønger et al., 1996). Upon excitation at 285 nm the intrinsic emission from Trp residues in PLA2 was monitored and the lag time was determined as the time elapsed between PLA2 addition and a sudden increase in fluorescence, which marks the onset of the reaction burst (Bell and Biltonen, 1991). The lag time measurements were performed in a temperature range for which the DMPC/1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) phase diagram indicates a two-phase coexistence region. It was found that PLA2 activity is highly non-trivial within this two-phase region, where two broad minima coincident with the solidus and the liquidus lines could be resolved (Høyrup et al., 2001). The presence of 1-stearoyl-2-myristoyl-sn-glycero-3-phosphocholine (SMPC) causes an increase in the lag time in the entire temperature range. A striking relationship was found between the thermotropic data in terms of transition temperature and heat capacity peak width on the one hand, and the PLA2 hydrolysis on the other hand. It is seen that the hydrolysis activity of PLA2 is modulated and lowered by inclusion of the asymmetric lipid SMPC into both one-component (1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)) or multi-component (DMPC/DSPC) lipid bilayers, suggesting a similar effect of SMPC.
on both systems (Høyrup et al., 2001). The results obtained in this study imply that SMPC is capable of lowering the interfacial tension between gel and fluid domains, thereby slowing down the interfacial dynamics.

The spectral properties of Trp-containing peptides in interaction with phospholipid bilayers can be further exploited through red edge excitation shift (REES) measurements. This term describes a shift in the wavelength of maximum fluorescence emission towards higher wavelengths, caused by a shift in the excitation wavelength towards the red edge of the absorption band of the chromophore (Suppan and Ghoneim, 1997). This effect is essentially observed with polar fluorophores in motionally restricted media, because it is due to the preferential excitation of the better solvated molecules that emit at longer wavelengths. If the effect is detected in the steady-state spectra of the fluorophore it means that solvent relaxation is slower than or at most occurs within the same time-scale as the emission process (Itoh and Azumi, 1975).

The interfacial region of the membrane is characterized by unique motional and polarity properties different from the bulk aqueous phase and the more isotropic hydrocarbon-like deeper regions of the bilayer and, in addition to an amphipathic environment, it offers the possibility of participation in intermolecular charge interactions and hydrogen bonding (White and Wimley, 1999). These structural features slow down the rate of solvent reorientation around the fluorophores and have been recognized as typical environments, which give rise to appreciable red edge effects (Itoh and Azumi, 1975). REES could therefore be a common feature in Trp residue emission, because they are in most cases located at the membrane/water interfacial region (see below).

An illustrative example of REES utilization in reporting peptide membrane binding is the work of Falls et al. (2001). The authors studied the interaction of synthetic peptides mimicking the N-terminal region of human prothrombin (PT) with phospholipid membranes by means of fluorescence techniques. The peptide PT-(1-46)F4W contains an additional Trp residue, by virtue of substitution of Phe at position 4, allowing fluorescence probing of the N-terminal segment. It was demonstrated in several vitamin K-dependent procoagulant proteins (e.g. factor IX; Freedman et al., 1996) that, following calcium binding, most of the Gla residues become internalized, resulting in solvent exposure of three hydrophobic residues within a calcium induced N-terminal loop (called o-loop). This loop has been implicated in the binding of vitamin K-dependent proteins to phospholipid membranes (Blostein et al., 2000). PT-(1-46)F4W has a maximum emission wavelength at 350 nm in buffer and no shift upon addition of 100% phosphatidylcholine (PC) small unilamellar vesicles (SUVs). Addition of egg PC/brain PS (4:1) originates a modest calcium-dependent blue-shift of 5 nm, and also an increase in fluorescence intensity. The REES effect is 0 nm for PT-(1-46), 0 nm for PT-(1-46)F4W in the presence of 100% PC SUVs, but 28 nm for this peptide in the presence of PC/PS SUV, pointing to a strong interaction of Trp-4 with PS-containing SUVs (Falls et al., 2001).

2.3. Time-resolved fluorescence measurements

As previously described, Trp fluorescence emission decay kinetics is usually complex, and in addition to a sum of exponentials (Eq. (4)), continuous distributions of lifetime populations have also been used to describe the fluorescence decays (e.g. Vincent et al., 2000). The complexity of single Trp peptides and proteins fluorescence decay kinetics can arise from two different but not mutually exclusive phenomena, namely the existence of ground state rotamers (Willis et al., 1994; Clayton and Sawyer, 2000) and solvent relaxation processes (Lakowicz, 2000; Toptygin et al., 2001). This complexity intrinsic to Trp emission decays precludes its utilization in the recovery of direct information about e.g., secondary structure in peptides/proteins (Ladokhin, 2001). However, structural information can still be recovered from time-resolved fluorescence data of Trp residues as illustrated in the following two studies.
Melittin is considered to be a paradigm for peptide–membrane interaction studies. The single Trp residue of this host-defense peptide present in bee venom plays a crucial role in maintaining the structure and hemolytic activity of membrane-bound melittin (Perez-Paya et al., 1994). Ghosh et al. (1997) studied melittin interaction with zwitterionic 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and anionic DOPC/1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG) or DOPC/1,2-dioleoyl-sn-glycero-3-phosphoric acid (DOPA) lipid vesicles. The fluorescence emission maximum of melittin incorporated into zwitterionic DOPC vesicles was around 336 nm (17 nm blue-shifted relative to buffer), and for anionic lipid containing vesicles it was 1–2 nm further blue-shifted. When bound to DOPC vesicles, this single-Trp peptide presents a REES of 5 nm, an effect that is not observed in buffer. On the other hand, in the presence of anionic DOPC/DOPG or DOPC/DOPA lipid vesicles, the REES effect was only of 2–3 nm. The average fluorescence lifetime (Lakowicz, 1999) of melittin in buffer is 1.85 ns ($\lambda_{\text{exc}} = 296$ nm; $\lambda_{\text{em}} = 330$ nm) and when bound to DOPC, DOPC/DOPG and DOPC/DOPA membranes it decreases to 1.23, 1.34 and 1.05 ns, respectively. In the membrane-bound form, melittin adopts a predominantly $\alpha$-helical conformation, for which the residues Trp-19 and Lys-23 are in close proximity. At physiological pH, the Lys-23 has a positive charge and this group is known to be an efficient quencher of Trp fluorescence, which explains the decrease in the fluorescence mean lifetime of melittin when going from aqueous to membrane-bound (Ghosh et al., 1997).

The fluorescence parameters of staphylococcal $\delta$-toxin (Trp-15 on the polar side of the amphipathic helix) and synthetic analogues with single Trp residues at positions 5 or 16 (on the apolar side) were studied by Talbot et al. (2001). $\delta$-Toxin is a membrane-active peptide of 26 amino acid residues with a strong tendency to aggregate in solution and therefore displaying maximum emission wavelength in aqueous environment characteristic of a moderately to strongly hydrophobic environment (Thiaudiére et al., 1991). It was shown by Talbot et al. (2001) that, despite the very small changes in the average polarity of the Trp residue microenvironment upon binding to PC/PS SUVs (4:1) detected by steady-state fluorescence, large modifications of the excited state population distributions of Trp, whatever its position, were observed. These changes were dependent on the hydrophilic/lipophilic position of Trp on the amphipathic helix.

### 2.4. Fluorescence anisotropy

Time-resolved, and to a lesser extent, steady-state fluorescence anisotropy measurements are powerful methodologies which give direct insight about the peptide rotational dynamics when in interaction with a membrane. Fluorescence anisotropy is defined by

$$r(t) = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities of the vertically ($I_{\parallel}$) and horizontally ($I_{\perp}$) polarized emission, when the sample is excited with vertically polarized light (Lakowicz, 1999). Fluorescence anisotropy decays are usually described as follows:

$$r(t) = (r_0 - r_{\infty})\exp(-t/\theta) + r_{\infty}$$

in which $\theta$ is the rotational correlation time, $r_0$ is the fundamental anisotropy, and $r_{\infty}$ is the limiting value of $r(t)$ for $t \to \infty$. From the latter parameter, restrictions to fluorophore rotation can be evaluated (Lakowicz, 1999). More than one rotational correlation time are usually required to describe the decay of the time-resolved anisotropy of a membrane-interacting peptide, and in case that they differ significantly, it is possible to appreciate both fast rotational dynamics of Trp residues, and a slower component due to the movements of protein (or peptide) segments.

Two recent works present methodologies of data analysis, showing the type of information that can be gained from these studies (Ayala-Sanmartin et al., 2000; Talbot et al., 2001). As an illustration, the more recent work is described below.

Talbot et al. (2001) carried out fluorescence anisotropy decay measurements of $\delta$-toxin and its single Trp synthetic analogues (Section 2.2) in...
aqueous solution and in interaction with lipid vesicles. In solution, the presence of peptide aggregates was detected through measurement of long or infinite rotational correlation times. However, upon binding to mixed PC/PS vesicles, this anisotropy decay component at infinite time disappeared ($r_{\infty} = 0$), and a slow rotational correlation time of 32 ns was detected for the peptide analogs with a Trp residue in the apolar side of the amphipathic helix (when the peptide was not highly associated in water), suggesting reorientation of the monomer in the membrane. It is also interesting to compare the barycenter of the fastest rotational correlation times measured for the ‘hydrophilic’ (Trp-15) and ‘hydrophobic’ (Trp-5 or -16) residues, because they correspond to local movements of Trp residues of the membrane-bound peptides. The fluorescence emission maxima of these peptides occur at 342 and 332 nm, respectively, and the fastest rotational correlation time is higher for the first than for the second one. Thus, the more superficial Trp residue is more immobilized than the one that penetrates deeper into the acyl chain region (Talbot et al., 2001).

As previously pointed out, a problem frequently encountered when the lipid/water partition coefficient of an amphipathic peptide is rather small is that even in the presence of a concentrated lipid suspension, the fraction of peptide remaining in the aqueous phase significantly contributes to the whole fluorescence emission of a sample. This implies that a detailed analysis of the fluorescence anisotropy decay of the peptide at shorter times is eventually too complex, because the fitting model would have to account for a too large number of parameters, i.e., it would have to describe at least two different peptide populations in a sample, each associated with a complex fluorescence intensity decay (described by two or three exponentials as previously indicated). However, the study of limiting anisotropies ($r_{\infty}$) of the peptide in the membrane is not hindered by this fact and their analysis can be carried out in a way similar to the one previously described for the correction of the fluorescence spectra using the following expression (Contreras et al., 2001):

$$r_{\infty}^L = \left(1 + \frac{x_W r_W}{x_L r_L}\right) r_{\infty}$$

(14)

where $r_{\infty}^L$ is the limiting anisotropy of the peptide when in the membrane and $r_{\infty}$ is the experimentally determined value for the highest lipid concentration. The latter value is always lower than $r_{\infty}^L$, due to contribution of non-polarized emission from the aqueous peptide. The other parameters are obtained from Eqs. (3) and (5). Experimental data obtained for α-MSH in interaction with a binary lipid system is depicted in Fig. 3, and the parameters obtained are shown in Table 1 (Contreras et al., 2001). As expected the limiting anisotropy for the peptide in buffer is $r_{\infty} = 0$, and upon interaction with the lipid the value increases up to $r_{\infty}^L = 0.13$. This high value for a non-transmembrane helix means that this peptide is strongly adsorbed at the membrane interface and highly immobilized. From this value the order parameter can be obtained (Lakowicz, 1999).

2.5. Fluorescence quenching studies

Trp residues buried in the vesicles hydrophobic core are shielded from the aqueous solution, and therefore upon membrane incorporation of the peptide an aqueous quencher should be less able to quench their fluorescence. Trp fluorescence quenching of peptides by acrylamide and iodide, which do not readily penetrate the membrane, is a

![Fig. 3. Anisotropy decays of α-MSH ($\lambda_{ex} = 295$ nm) in buffer (A) and in the presence of 5.2 mM of DMPC/DMPA (3:1) LUV at 37°C (B). Reprinted from Contreras et al., 2001, with permission. Copyright 2001 Biophysical Society.](image-url)
popular methodology and was applied to the previously described systems PT-(1-46) and PT-(1-46)F4W (Falls et al., 2001). For the highest quencher concentration used, KI produced an 80% reduction in the fluorescence intensity of the peptides in buffer solution. Addition of lipid reduced in the fluorescence intensity of the (fractional accessibility 0.63) and K1-46F4W (Falls et al., 2001). For the highest pre popular methodology and was applied to the

...ing that Trp accessibility to the quencher did not vary. However, addition of PC/PS vesicles reduced the quenching to only 45% in the case of PT-(1-46)F4W. Quenching may be examined quantitatively by the Stern–Volmer plot, which relates fluorescence quenching to the concentration of quencher [Q] by the equation I0/I = 1 + KSV[Q], where I0 is the fluorescence intensity of the peptide measured in the absence of the quencher, I is its fluorescence intensity at a given quencher concentration, and KSV is the Stern–Volmer quenching constant. Stern–Volmer plots for I− quenching of PT-(1-46)F4W were linear in the absence of lipid, but not in the presence of PS-containing SUVs. Considering two populations of fluorophore, Stern–Volmer constants KSV = 9.0 ± 2.0 M−1 (fractional accessibility 0.63) and KSV ≈ 10−5 M−1 were obtained. The higher KSV value probably corresponds to the water-accessible Trp-4 in whereas the other one stems from the membrane-buried Trp-4. It should be stressed that the binding of vesicles per se increases I0, so it is not a decrease in lifetime that leads to this low KSV value.

Another useful strategy to gain information about the transverse location (degree of penetration) of the membrane-bound peptide molecules is to use spin-labeled or brominated fatty acids or phospholipids in depth-dependent fluorescence quenching experiments (for a recent review, see London and Ladokhin, 2002). These molecules, derivatized at different positions along their acyl chains, are known to insert predominantly vertical to the bilayer surface of the lipid vesicles. Therefore, their quencher groups are located at different depths in the lipid bilayer, allowing information about the Trp residue location in the lipid vesicles to be obtained, because the highest quenching efficiencies of membrane-bound peptides should be observed when the quencher moiety is closest to the Trp fluorophore. When using spin-labeled fatty acids one should take into consideration their quencher group position-dependent membrane/water partition coefficients. In the present context we will only describe qualitative applications of this methodology or refer to results obtained after application of the parallax method (Chattopadhyay and London, 1987).

For PT-(1-46)F4W (see above) the estimated depth for Trp-4 was 5–7 Å (for PT-(1-46), there was very little fluorescence quenching by all quenchers used). Considering a transverse width of the indole ring ≈ 5.5 Å and that the interfacial membrane region extends ≈ 5 Å into the membrane from the lipid/water interface, the Trp should be located within the shallow interfacial region of the membrane (Falls et al., 2001). Based on these results (see also blue-shift and REES data in the respective sections) the authors conclude that hydrophobic residues within the ω-loop of the PT Gla domain selectively interacted with PS-containing vesicles, providing a membrane anchor. A similar mechanism possibly also applies to other proteins, e.g., C2 domain of factor VIII and factor V, that share a conserved β-barrel framework with 3 protruding loops that contain a group of solvent exposed hydrophobic residues.

Returning to melittin, Ghosh et al. (1997) also proposed an interfacial localization for its Trp residue in its membrane-bound form after the application of the parallax method with spin-labeled PCs. Interestingly, the Trp depth was 11 Å for all the lipid vesicle compositions used in this work. The rationalization of all the fluorescence data obtained in this study (partly described in a previous section) was that the main difference between zwitterionic and anionic vesicles was the water penetration induced by melittin. In the first case, the establishment of hydrophobic interactions is essential for peptide binding, leading to a stronger perturbation of the membrane and a deeper penetration of water molecules, which are immobilized relative to the bulk water. This leads to a less hydrophobic environment (smaller blue-shift) but a stronger REES effect. The strong electrostatic interactions between the peptide and the anionic phospholipid headgroups, on the other hand, inhibit the lytic activity of the peptide
preventing the formation of a putative second type of membrane-bound structure.

Fluorescence of entrapped carboxyfluorescein (high self-quenching concentration) increases upon its release from the liposomes interior. This can be used to monitor the lytic activity of peptides. Initially, a low background fluorescence is observed in all cases, but upon addition of, for example, melittin, an increase of carboxyfluorescein fluorescence due to the permeabilization of the vesicles was observed by Ghosh et al. (1997). The maximum lytic efficiency calculated relative to that of TX-100 (complete disruption of the vesicles) was for zwitterionic vesicles (DOPC) of 80%, whereas for mixed vesicles (either 40% DOPC/60% DOPG or 50% DOPC/50% DOPA, mol/mol) it was reduced to 25–30%. These results agree with the model of interaction described above.

2.6. Trp/probe FRET studies

A particularly powerful fluorescence technique to probe lipid composition in the vicinity of the peptide (and thus the latter’s selectivity to a certain lipid type) is fluorescence resonance energy transfer (FRET). The kinetics of FRET was originally derived by Förster (1949). The rate of energy transfer between a donor molecule, with fluorescence lifetime \( \tau \), and an acceptor molecule, separated by a distance \( R \), is given by,

\[
k_T = \frac{1}{\tau} \left( \frac{R_0}{R} \right)^6
\]

(15)

where \( R_0 \) is the critical distance, which can be calculated from

\[
R_0 = 0.2108 \left[ \kappa^2 \Phi_D n^{-4} \int_0^{\infty} I(\lambda) \varepsilon(\lambda) \lambda^4 d\lambda \right]^{1/6}
\]

(16)

where in turn \( \kappa^2 \) is the orientation factor (see Van der Meer et al., 1994, for a detailed discussion), \( \Phi_D \) is the donor quantum yield in the absence of acceptor, \( n \) is the refractive index, \( \lambda \) is the wavelength, \( I(\lambda) \) is the normalized donor emission spectrum, and \( \varepsilon(\lambda) \) is the acceptor molar absorption spectrum. In Eq. (15), if the \( \lambda \) units used are nm, the calculated \( R_0 \) has Å units. The kinetics of FRET from a donor to an ensemble of acceptors (the situation for membrane experiments) is presented in more detail in the following section. At this point, examples from the literature of simple FRET experiments that use Trp from a peptide as donor and a lipophilic probe in a binary lipid bilayer as acceptor will be described. Because of the relatively complex formalism and limited availability of state of the art time-resolved fluorescence instrumentation, most of these works are essentially of a qualitative nature, often based on steady-state measurements of the efficiency of energy transfer (see below Eq. (22)). Pedersen et al. (2001) measured Trp fluorescence from a small positively charged peptide with a N-terminally linked acyl chain to vesicles of either pure DPPC or DPPC/1,2-dipalmitoyl-sn-glycero-3-phosphoserine (DPPS) (7:3), labeled with dansyl-labeled-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) (acceptor), and concluded that the peptide had higher binding affinity towards the lipid system containing the negatively charged DPPS lipids. Polozov et al. (1997a) studied the interaction of two peptides, GIKKFLGSWKFI-KAFVG (18L, cationic) and N-acetyl-DWLKAFYDKVAEKLKEAF-amide (Ac-18A-NH2, zwitterionic overall, but bearing positive charges at the protein–lipid interface) with binary lipid systems. These peptides feature the archetype sequences of the L- (lytic) and A- (apolipoprotein) type sequences, respectively (Segrest et al., 1990). In this work, the authors measured FRET between the Trp residue of 18L or Ac-18A-NH2 and anthrylvinyl-labeled phospholipids 1-acyl-2-[trans-12-(9-anthryl)-11-dodecenoyl]-sn-glycero-3-phosphocholine (APC), -phosphoethanolamine (APE), and -phosphoglycerol (APG) with a value of \( \sim 24 \) Å for \( R_0 \). The lipid systems were DMPC/DMPG (37 °C, fluid membranes in the absence of peptide) and DOPC/1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) equimolar mixtures. Regarding the DMPC/DMPG system, for both peptides the FRET efficiency to APG was significantly higher than to APC, which indicates that both peptides are preferentially associated with acidic lipids. The observation that no large-scale phase separation occurs in the phosphatidylglycerol (PG)/PC system (as verified from the FRET experiments between APC or APG and
PPC (1-acyl-2-[9-(3-perylenoyl)nonanoyl]-sn-3-glycerophosphocholine), see below) implies that this association is limited to the peptide close vicinity. This effect was not observed in the DOPC/DOPE zwitterionic mixture, for which the efficiency values were identical for the peptide/APC and peptide/APE pairs. Interestingly, whereas in the DMPC/DMPG system FRET efficiencies were higher for the 18L/probe pairs than for the Ac-18A-NH₂/probe pairs (indicating, according to the authors, increased depth of incorporation of 18L), for DOPC/DOPE the measured values for the two peptides were identical.

Other uses of peptide/probe FRET (which take advantage of its dependence on the acceptor concentration) include assays for peptide translocation (Matsuzaki et al., 1995; see Drin et al., 2001, for an application to binary mixtures).

3. The bilayer point of view: fluorescence spectroscopy approaches based on membrane probes

Being natural phospholipids not fluorescent, lipophilic fluorescent probes must be used in order to study the effect of incorporation of peptides on the lipid organization using fluorescence techniques. Some useful approaches are described below.

3.1. Fluorescence anisotropy

Common membrane probes (for a review, see Davenport, 1997) display different rotational dynamics during the excited state for distinct lipid environments (gel phase, fluid phase, liquid ordered phase). Especially useful are the probes that have (i) large difference in steady-state anisotropy for different lipid phases; (ii) partition coefficient close to unity for phase-separated systems; and (iii) similar values of fluorescence quantum yield in different lipid phases. DPH fulfils all these conditions, and has been used extensively to monitor the order and dynamics within the acyl chain region of liposome lipid bilayers (Lentz, 1993). DPH fluorescence anisotropy decreases clearly with increasing temperature in the range of gel/fluid phase transition (for one-component systems), as illustrated in Fig. 4, and this has been exploited in the determination of phase diagrams for binary lipid mixtures (Lentz et al., 1976). Its use in relation to peptide interaction with binary lipid mixtures is more limited, and mostly qualitative information has been obtained by comparing the variation of probe polarization with temperature before and after peptide addition.

Thus, Sixl and Galla (1981) used the alterations in the profile of variation of DPH fluorescence anisotropy with temperature to show that the binding of the cationic polypeptide antibiotic polymyxin B to negatively charged 1,2-dipalmitoyl-sn-glycero-3-phosphoric acid (DPPA)/DSPC 1:1 mixtures (these lipids have approximately the same main transition temperature, 53–54 °C, in the conditions of the experiment) in a 1:2 peptide:DPPA mole ratio leads to phase separation.
with formation of polymyxin-bound phosphatidic acid domains. Theretz et al. (1983) studied the interaction of this same peptide with both the negatively charged lipid 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG) alone and DMPC/DPPG mixtures, and verified that it can also induce phase separation in these systems. DPH fluorescence anisotropy was also used by Lafleur et al. (1989) to study the effect of melittin on different binary mixtures of phospholipids. For DPPC/DSPC, no phase separation occurs in an equimolar mixture of these lipids in presence of melittin (very large amounts of the peptide must be added to detect significant changes in the variation of DPH polarization with temperature). However, when DPPG is mixed with either DPPC or DSPC, the addition of melittin leads to phase separation.

Tendidian and Lentz (1990), using also DPH fluorescence anisotropy, determined the phase diagram of the DMPC/1,2-dimyristoyl-sn-glycero-3-phosphoserine system in the presence and absence of bovine PT. The shape of the mixture phase diagram was essentially unaltered by the binding of PT in the presence of Ca\(^{2+}\) although the gel/fluid coexistence region was slightly narrowed and shifted to higher temperatures. Taking this into account, together with absolute anisotropy values, the authors concluded that instead of implying domain formation, their results demonstrate that the observed phase diagram shifts can be accounted for by changes in membrane order both in the fluid and in the gel phases as a result of PT binding.

Gawrisch et al. (1993) studied the interaction of the peptide RVIEVQGACRAIRHIPRRRIR (P828; derived from the C-terminus of HIV gp160, and with high positive charge) with DOPC/DOPG membranes. They verified that both APCs and APGs polarization increases (from 0.050 to 0.066, and from 0.073 to 0.162, respectively) upon addition of P828 to DOPC/DOPG 4:1. These values indicate that in average both probes sense a more rigid environment, possibly due to formation of DOPG-rich ordered domains, as a result of peptide binding. The fact that APGs anisotropy undergoes the most significant increase is justifiable assuming that this probe has large affinity for these clusters, as should be expected.

In the previously mentioned work of Polozov et al. (1997a), the steady-state anisotropy of APC, APE and APG was measured in mixtures including DMPG/DPPC, DMPC/DPPG, DMPC/1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMEP) and DMPC/DPPE. For the DMPG/DPPG system, in the absence of peptide, both APC and APL report gel/fluid phase coexistence in the 24.5–33 °C range. Addition of Ac-18A-NH\(_2\) broadens this range to 24–36 °C, indicating that the gel becomes depleted of the low-melting acidic lipid upon addition of peptide. For the DMPC/DPPG system, the phase coexistence range is broadened from 30–32.5 °C to 27–33 °C, indicating that the fluid becomes depleted of the high-melting acidic lipid upon addition of peptide. Moreover, for this system, the anisotropy of both APC and APG is lower in the presence of peptide, and according to the authors this illustrates that preferential association of peptides with negatively charged lipids can decrease the stability of the gel. Addition of 18-L leads to similar results.

For the zwitterionic mixtures DMPC/DMPE and DMPC/DPPE, the APC and APE probes report the phase transition differently, probably as a consequence of a more extensive gel (rich in phosphatidylethanolamine)/fluid (rich in PC) phase separation. Interestingly, addition of 18-L to DMPC/DMPE mixture results in a coincidence of the variation of anisotropy of both probes with temperature, which could point to an increase of miscibility of the system (possibly a reduction of domain size).

### 3.2. Laurdan generalized polarization

2-Dimethylamino-6-lauroylnaphtalene (Laurdan) is a fluorescent probe which exhibits a strong red-shift in its emission for high polarity solvents. When incorporated into phospholipid bilayers, Laurdan emission depends strongly on the lipid phase, having maxima close to ~490 nm in the fluid phase and ~440 nm in the gel phase. An operational parameter, known as ‘generalized polarization’ (GP) may be loosely defined as

\[
GP = (I_g - I_f)/(I_g + I_f),
\]

where \(I_g\) and \(I_f\) are the...
excitation/emission intensities, at fixed emission/ excitation wavelength (for the calculation of emission/excitation GP, respectively), at wavelengths close to the maxima in the gel and fluid, respectively (Parassassi et al., 1990). Experimental GP values are typically high for ordered phases (e.g. \( \sim 0.5 \)) and low (negative) for disordered phases. In a recent work, Granjon et al. (2001) studied the interaction of the protein mitochondrial creatine kinase (mtCK) with DMPC/DMPG liposomes, concluding (by comparison of the variation of excitation GP with temperature in the presence and in the absence of protein, and by measuring excitation and emission GP spectra) that the effect of mtCK binding is an increase in membrane order.

3.3. Probe–probe fluorescence resonance energy transfer

FRET is a convenient tool to study lipid phase separation. In case the donor molecules are surrounded by a distribution of acceptors, the decay law becomes complex (Loura et al., 2001). The result for an infinite two-dimensional system (or any system with donor and acceptor in the same plane—cis transfer), assuming that there is no homotransfer between donors, the fraction of excited acceptors is negligible, translational diffusion is negligible, and the exclusion distance between donors and acceptors is much smaller than \( R_0 \), is

\[
i_{DA,\text{cis}}(t) = \exp(-t/\tau - C(t/\tau)^{1/3})
\]

where

\[
C = \Gamma(2/3)n\pi h_0^2
\]

In this equation, \( n \) is the surface density of acceptors and \( \Gamma \) is the complete gamma function. If donors and acceptors are located in infinite parallel planes (trans transfer, as often occurs in bilayers for chromophores located at different depths), separated by a distance \( h \), the result is (Davenport et al., 1985):

\[
i_{DA,\text{trans}}(t) = \exp(-t/\tau - kh^2 CF(h, t))
\]

where

\[
F(h, t) = \int_0^1 \frac{1 - \exp\left(-t/\gamma \left(R_0/h\right) \gamma x\right)}{x^2} dx
\]

and \( \gamma = 2/R_0^2 \).

Membrane phase separation leads to partition of both donor and acceptor probes between the two phases. In general, the donor fraction and fluorescence properties will be different in the two phases, as will be the local acceptor concentrations. The overall donor fluorescence decay will be a linear combination of the decay in each phase (which will be given by one of the above equations), weighed by the respective amount of donor molecules (proportional to the \( A \) factors in the following equation):

\[
i_{DA,\text{phase separation}}(t) = A_1 i_{DA,\text{phase 1}}(t) + A_2 i_{DA,\text{phase 2}}(t)
\]

In all cases, the FRET efficiency (readily obtained from steady-state experiments and useful as a quick indicator) is defined by

\[
E = 1 - \int_0^\infty i_D(t) dt / \int_0^\infty i_{DA}(t) dt
\]

where \( i_D(t) \) is the donor decay in the absence of acceptor. When \( E \) is obtained from time-resolved experiments, the integration can be carried out analytically, but especially for trans transfer or multiexponentially decaying donors (as is often the case in model membranes) is best done numerically.

As an illustration of the potential of FRET between membrane probes in the study of the effect of peptides on the organization of binary lipid systems, three examples will be considered. The first of these is a qualitative study carried out by Gawrisch et al. (1993). These authors report a slight decrease in \( E \) between anthrylvinyl-labeled-PG and perylenoyl-labeled PC in DOPC/DOPG 4:1 mixtures as a result of addition of P828, interpreted as indicating formation of DOPG-rich clusters (and poor in acceptor probe).

The second example comes from the aforementioned work by Polozov et al. (1997a). These authors titrated, with the two peptides (18L and Ac-18A-NH₂), equimolar DOPC/DOPG fluid phase mixtures, doped with APC or APG as
donors and PPC as acceptor ($R_0 = 44 \text{ Å}$), and measured the variation of FRET efficiency. For both APC/PPC and APG/PPG pairs there was a decrease in $E$, which was interpreted as resulting from the increase in total surface area upon peptide incorporation. From these measurements, the authors were able to compare the area occupied by the two membrane-inserted peptides. The observation that the relative decrease in $E$ was the same for both pairs suggests that no large-scale ($> R_0$) phase separation is taking place upon peptide incorporation.

As a final example, we will describe briefly our study of the interaction of the polylysine peptide $K_6W$ with equimolar DPPC/DPPS LUVs. Because of the high positive charge of the peptide, reorganization of the zwitterionic lipid (DPPC)/acidic lipid (DPPS) mixture was expected. This could be detected by the variation of FRET efficiency between fluorescently labeled DPPC analogs upon incorporation of peptide. To this extent, $2\text{-}[3\text{-}(\text{diphenylhexatrienyl)}\text{propanoyl}]\text{-1-hexadecanoyl-sn-glycero-3-phosphocholine (DPH-PC) was used as donor and 1-palmitoyl-2\text{-}[12\text{-}(7\text{-nitrobenz-2-oxa-1,3-diazol-4-yl)}\text{aminododecanoyl}]\text{-sn-glycero-3-phosphocholine (NBD-PC) was used as acceptor ($R_0 (45 \text{ °C}) = 41 \text{ Å}; R_0 (60 \text{ °C}) = 40 \text{ Å}$). Two distinct situations were considered: at $T = 60 \text{ °C}$, above the main transition temperature ($T_m$) of both phospholipids, there is a single fluid lipid phase in the absence of peptide; at $T = 45 \text{ °C}$, between the $T_m$ values for DPPC and DPPS, there is gel/fluid phase coexistence in the absence of peptide. We sought to determine whether the insertion of this basic peptide would lead to: (i) phase separation from a single fluid phase DPPC/DPPS mixture ($T = 60 \text{ °C}$) and, (ii) noticeable reorganization of pre-existing gel/fluid domains ($T = 45 \text{ °C}$). The former effect would be clearly apparent in a FRET measurement, as segregation of a DPPC-rich phase (containing most of the probe molecules) would lead to local enrichment of acceptor probe, and thus higher FRET efficiency. The latter effect would be visible for example if the pre-existing domains were very small ( ~ 10 nm or smaller), and became considerably larger upon incorporation of peptide (in practice, this would also correspond to an increase of FRET efficiency, because the probe distribution for very small domains is similar to that of a one-phase system, while that of larger domains is closer to the complete phase separation limit).

As a first approach, fluorescence intensity decays were described by empirical sum-of-exponentials functions (Eq. (4)) and the recovered fitting functions were integrated to calculate the FRET efficiency $E$ (Eq. (22)). The value obtained for this parameter at 60 °C in the absence of peptide is approximately 0.40, close to the theoretical expectation for a random distribution of acceptors (0.43), which can be computed from Eqs. (19), (20) and (22). In this calculation, $h = 1.7$ nm (corresponding to half the bilayer width (Marsh, 1990)) was considered, because, whereas it is a good approximation to consider the DPH moiety of DPH-PC located in the center of the bilayer, the NBD group of NBD-PC snorkels to the bilayer interface (Chattopadhyay and London, 1987; see Fig. 5, left). When peptide is added, $E$ increases up to 0.54 (for a peptide:lipid ratio of 1:13), seeming to indicate phase separation. On the other hand, addition of peptide to the gel/fluid mixture (45 °C) does not lead to a significant increase in $E$, i.e., there was no detectable rearrangement of the pre-existing lipid domains.

![Fig. 5. Schematic representation of the formation of multibilayers from DPPC:DPPS fluid LUV in the presence of $K_6W$, as revealed from the time-resolved fluorescence data analysis (see text for details).](image-url)
Despite being a rapid way of obtaining a first glimpse into the possible membrane organization, the steady-state analysis information is always limited. The FRET efficiency parameter results from time integration of the actual decay law, and in this process considerable detail regarding the probes distribution is lost. Thus, whenever possible, a complete parameter analysis of the time-probes distribution is lost. When carried out (ideally with global analysis of samples with and without acceptor), as reviewed recently (Loura et al., 2001). In this experiment, analysis of the decays recorded at 45 °C was made using Eq. (21) (together with Eqs. (19) and (20)), and from the essentially invariant recovered parameters, it was possible to conclude that addition of peptide does not indeed lead to appreciable changes in domain organization. On the other hand, it was not possible to analyze the decays recorded at 60 °C in the presence of peptide with the same model. For these samples, a different regime was observed in the decay profile: at longer times, the decay in presence of acceptor did not become parallel (in a log scale) to that without acceptor, as it should according to Eqs. (19) and (20). Successful analysis required the use of a modification of Eq. (19), allowing for FRET to a second layer of acceptors:

\[ I_{DA,\text{trans}}(t) = \exp(-t/\tau) - kC(h_1^2 F(h_1, t) + h_2^2 F(h_2, t)) \]  

(23)

In this equation, \( h_1 \) and \( h_2 \) are now the distances between the plane of the donors and the two distinct planes of acceptors. When \( h_1 = 1.7 \) nm was assumed (see justification above), the most successful analysis was obtained for \( h_2 = 4.1 \) nm. No evidence for phase separation was apparent from the decay analyses.

A possible explanation for these results is that the value recovered for \( h_2 \) corresponds to FRET to acceptors located in a different lipid bilayer to that of the donors. In this scenario, \( K_w \) promotes bilayer aggregation of DPPC/DPPS bilayers LUV in the fluid phase, possibly leading to formation of small anionic lipid clusters within each bilayer, but without leading to detectable phase separation (Fig. 5, right). This finding agrees with a microscopy study of Murray et al. (1999) who also ruled out the induction of large domains by basic peptides in mixtures containing acidic lipids.

Of course, in the framework of our model, there would be at least a third layer of acceptors located at approximately \( h_3 = (h_2 + \text{one bilayer width}) \approx 7.5 \) nm. However, the contribution of FRET to this plane (and further planes) of acceptors would be much smaller and effectively masked by FRET to acceptors located at \( h_1 \) and \( h_2 \). In the gel/fluid coexistence range, there is already gel/fluid phase separation in the absence of the peptide, and its addition probably leads to its incorporation preferably in the DPPS-rich gel domains, without significant rearrangement.

FRET measurements can also be used in lipid-mixing assays, as originally developed by Struck et al. (1981). In a possible setup, vesicles labeled with donor and acceptor probes are mixed with unlabeled vesicles. If the probes do not transfer spontaneously between vesicles, lipid mixing (as a result of vesicle fusion) results in dequenching of donor fluorescence (due to reduction in acceptor concentration). Of course, this methodology can be applied to the study of peptide-induced fusion. For example, Polozov et al. (1997b) confirmed fusion of DOPC/DOPE 1:1 vesicles induced by 18L using the APC/PPC FRET pair.

3.4. Other fluorescence spectroscopy methodologies

Formation of excimers of pyrene-labeled probes is a useful, albeit seldom explored, fluorescence approach for detection of phase separation. Hinderliter et al. (2001) studied the effect of adding C2 protein motif to a binary mixture of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, and 1-palmito-2-oleoyl-sn-glycero-3-phosphoserine (POPS), containing 4% of a pyrene-labeled anionic phospholipid (1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol). Most proteins with C2 domains function in signal transduction or membrane traffic, and the isolated C2 domains are autonomously folding modules that can function as Ca\(^{2+}\)-dependent phospholipid binding proteins (Rizo and Südhof, 1998). In the work by Hinderliter et al. (2001), as the C2 motif concentration increases, the excimer/monomer
(E/M) ratio (emission at 396 nm/470 nm) of the pyrene fluorescence first increases and then decreases. These data were rationalized using Monte Carlo simulations, and the results indicate the existence of a maximum in PS domain size as a function of protein concentration. The authors conclude that lipid and protein clustering are highly correlated, as a result of thermodynamic coupling between the intrinsic tendency for lipid clustering in the absence of any protein–protein interactions and preferential binding of the protein to POPS.

In another recent work, Zhao et al. (2002) studied the interaction of temporins with 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC)/POPG vesicles. Temporins are short (10–13 amino acids) linear antimicrobial peptides first isolated from the skin of the European red frog, with net positive charge and an amidated C-terminus (Simmaco et al., 1996). They potentially assume an amphipathic \( \alpha \)-helical conformation in apolar solvents such as trifluoroethanol and permeabilize lipid vesicles irrespective of their composition (Mangoni et al., 2000). The observed increase in \( E/M \) ratio of 1-palmitoyl-2-[10-(pyren-1-yl)decanoyl]-sn-glycero-3-phosphocholine fluorescence by Zhao et al. (2002) could be a result of two non-mutually exclusive mechanisms: segregation of pyrene-labeled PC into microdomains and increase in lateral mobility of the lipids. Both temporins B and L caused only minor changes in DPH anisotropy for SOPC LUVs. However, with increasing POPG fraction, a progressive increase in acyl chain order was apparent from DPH anisotropy, and so the increase in \( E/M \) can also be interpreted as lateral segregation of lipids (resulting in a clustering of pyrene-labeled PC). The formation of anionic lipid and enriched peptide domains is, according to the authors, related to pore formation for which peptide aggregation is necessary, because the negative charge of the lipid reduces the unfavorable electrostatic interaction between cationic peptides.

Another approach, the self-quenching of probe fluorescence, may also be a qualitative indicator of lipid domain formation. In the aforementioned work by Gawrisch et al. (1993), the authors report that DOPC/DOPG mixtures containing 20 mol% of the latter component and 1 mol% of APG show significant self-quenching of fluorescence after addition of P828, possibly as a result of lipid cluster formation.

4. Conclusions

In this work, several fluorescence methodologies that give insight on structural details of peptides in interaction with binary phospholipid mixtures (with emphasis on zwitterionic and negative lipids) are described.

Experiments can be designed based on the peptide intrinsic fluorescence, essentially to monitor the interaction and characterize the peptide environment, or using suitable membrane probes that report membrane structural alterations upon peptide interaction.

Direct inspection of fluorescence steady-state data allows to qualitatively conclude about 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 dynamics as well as topology (e.g. vesicle interaction and phospholipid lateral distribution).

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