Detection of Phospholipid Bilayers Microheterogeneity and Domains by Fluorescence Spectroscopy

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The goal of our studies is the determination of the molecular dynamics of the lipid matrix of membranes. A better knowledge of the lipids dynamics can be of relevance in understanding the membrane's role in the modulation of the cell life. The partition and the activity of membrane proteins and activity can be regulated by the physical state of the surrounding lipids (1).

Our investigations have been mainly focused in determining how the complex and heterogeneous composition of the membranes lipids can originate averaged physical properties, the coexistence of distinct properties, and the dynamics of the fluctuation between them. Referring to the gel and to the liquid-crystalline phase of the bilayer, the coexistence of those two different phases in a complex lipid matrix can be regarded as phase domain coexistence (2-4).

For our investigations we utilized the properties of the fluorescence emission of some membrane probes. The attractiveness of fluorescence spectroscopy for membrane studies lies in the high sensitivity of the technique, so that the perturbing effect of the probe on membrane structure is virtually null, in the sensitivity of fluorescence parameters to the physical properties of the environment, and in the intrinsic time scale of the fluorescence phenomena, typically in the range of nanoseconds, that is considered the timescale of most membrane dynamics.

We will report below some recent results obtained by using two membrane probes, DPH (1,6-diphenyl-1,3,5-hexatriene) and Laurdan (2-dimethylamino-6-lauroylphthalene).
Sensitivity of DPH to membrane microheterogeneity

Among all membranes probes, DPH is perhaps the most popular (5). DPH possesses favourable properties such as the hydrophobic character, the high quantum yield in membrane and negligible in water, the high sensitivity of the polarization and lifetime values to the phospholipid phase state. DPH fluorescence decay in phospholipids and in cell membranes has been best described by a continuous lorentzian distribution of lifetime values (6-7). The center of DPH lifetime distribution in vesicles composed of pure gel and in pure liquid-crystalline phase is around 10 ns and 7 ns, respectively. The full width at half maximum (that in the followings will be simply reported as width) is of about 0.6 ns in the gel phase, decreasing to a minimum of about 0.05 ns in the liquid-crystalline phase. In mixed phase phospholipids, the center shows an intermediate value and the width of the distribution is generally quite broad. A further short-lived component (around 3 ns), with low fractional intensity (<2%), has also been observed both in single and in mixed phase vesicles.

In several cell membranes the measured DPH lifetime distribution is generally broad, with a width varying as a function of the physiological state of the cell (7-9), and with a center value generally intermediate between those determined in liposomes for the pure gel and the pure liquid-crystalline phase. The distribution of DPH lifetimes observed in cell membranes has been attributed to a local heterogeneity sensed by the probe (7) and the width value has been utilized to evaluate the variation of the cell membranes "microheterogeneity" in different physiological conditions. Nevertheless, the reason of the center variation and the physical origin of the lifetime distribution remained unclear, as well as the origin of the short-lived component of the DPH decay (10), always observed in phospholipid vesicles and in natural membranes.

A photophysical model for the DPH decay has been recently proposed (11). The model is based on two separate, interconverting states (Figure 1). The first is populated upon absorption and is higher in energy than state 2. From the state 1 DPH molecules can decay with a high rate (short lifetime value), of about 0.7 x 10^9 s^-1, or can interconvert to state 2, with a rate R21. From state 2 originates most of the observed DPH fluorescence, characterized by a lower decay rate, of about 0.06 x 10^9 s^-1. The relative observation of one specie with respect to the other is contained in the parameter named species associated spectrum (SAS), the relative superposition of the two states, and for DPH molecules decaying from state 2 the value of SAS2 resulted of 0.47 for all samples examined. This result implies that the two states have essentially the same spectrum. Also a backward interconversion rate, from state 2 to state 1, R12, is allowed by the model. The two-state model well fits the DPH decay data obtained by multifrequency phase and modulation technique in various phospholipids and in a number of pur R12 are sensitive to by using the Global several experiment.
Fig. 1. Photophysical model for DPH decay. Excitation only occurs from state 1. R1 and R2 are the decay rates from state 1 and state 2, respectively. R21 and R12 are the interconversion rates between the two states. SAS1 and SAS2 represent the relative observation of the emission from state 1 and state 2, respectively. B1 and B2 are the initial population of state 1 and state 2, respectively. In the fit, their values have been fixed to B1 = 1 and B2 = 0.

In a number of pure organic solvents, both interconversion rates R21 and R12 are sensitive to solvent and temperature effect. Data have been analysed by using the Global method (12) which allows the simultaneous analysis of several experiments performed at different temperatures. The results show
that most of the observed features of the DPH decay are due to variations of
the backward interconversion rate, R12. The ratio between the rates R21/R12, which determines the value of the observed lifetime, strongly
depends on the polarity of the environment (Table I). Moreover, R12 is very

**TABLE I: Forward (R21) and backward (R12) interconversion rates between state 1 and state 2 of DPH in solvents. \(< \tau >\) is the averaged lifetime. The R12 values at different temperatures are reported. The ratio between the two interconversion rates is also reported (R21/R12). Rates values are expressed as \(10^6 \text{s}^{-1}\) and lifetimes as \(10^3 \text{s}^{-1}\).**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>R21</th>
<th>R12</th>
<th>(&lt; \tau &gt;)</th>
<th>R21/R12</th>
</tr>
</thead>
<tbody>
<tr>
<td>dodecane (T C: 10 - 40)</td>
<td>3.85</td>
<td>0.012-0.09</td>
<td>12.7</td>
<td>42.79</td>
</tr>
<tr>
<td>cyclohexane (T C: -8 - 40)</td>
<td>4.46</td>
<td>0.14-0.11</td>
<td>13.2</td>
<td>44.00</td>
</tr>
<tr>
<td>tetrahydrofuran (T C: 0 - 40)</td>
<td>4.25</td>
<td>0.40-0.33</td>
<td>9.5</td>
<td>12.60</td>
</tr>
<tr>
<td>chloroform (T C: -28 - 30)</td>
<td>3.70</td>
<td>0.97-0.77</td>
<td>6.2</td>
<td>4.80</td>
</tr>
</tbody>
</table>

sensitive to temperature and to phospholipids phase state, so that its variation
is at the origin of the lifetime change in the different phases. In our model,
the short-lived component is due to the small fraction of DPH molecules
decaying from state 1. For the data obtained in phospholipids, the value of
the rate R12 resulted distributed, reintroducing the concept of microheterogeneity (Table II). The width of the R12 distribution decreases
dramatically by passing from the gel to the liquid-crystalline phase of
phospholipids, in good agreement with the previously measured lifetime in
phospholipid vesicles.

A further consequence, deduced from the above photophysical model,
concerns the possibility of resolving phase domains coexistence in
phospholipid bilayers. From the values reported in Table II we can observe
that the variation of R12 center and width in the two phases is relatively
small and, more relevant, that its width is a relevant part of the center value.
Hence, an intermediate specie will be very difficult to detect and we
conclude that the use of DPH is not appropriate for the detection of phase
domain coexistence in membranes of unknown composition. However, since
DPH lifetime is very sensitive to the polarity of the environment, variations
of the width of its lifetime distribution can be profitably used to estimate the
variation of microheterogeneity of cell membranes caused by different
physiological and pathological conditions (7-9).
TABLE II: Forward (R21) and backward (R12) interconversion rate versus temperature of DPH excited state dipalmitoyl-phosphatidylcholine vesicles. The R12 rate was distributed using a lorentzian function of full width at half maximum (W) are reported.
Rate values are expressed as 10^6 s^-1.

<table>
<thead>
<tr>
<th>T(C)</th>
<th>R21</th>
<th>R12</th>
<th>W</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>3.84</td>
<td>0.43</td>
<td>0.29</td>
</tr>
<tr>
<td>9.8</td>
<td>3.87</td>
<td>0.40</td>
<td>0.28</td>
</tr>
<tr>
<td>14.0</td>
<td>3.97</td>
<td>0.41</td>
<td>0.23</td>
</tr>
<tr>
<td>21.6</td>
<td>4.05</td>
<td>0.39</td>
<td>0.24</td>
</tr>
<tr>
<td>32.5</td>
<td>4.07</td>
<td>0.36</td>
<td>0.22</td>
</tr>
<tr>
<td>36.0</td>
<td>4.24</td>
<td>0.36</td>
<td>0.25</td>
</tr>
<tr>
<td>39.0</td>
<td>4.28</td>
<td>0.34</td>
<td>0.31</td>
</tr>
<tr>
<td>40.5</td>
<td>4.21</td>
<td>0.52</td>
<td>0.29</td>
</tr>
<tr>
<td>44.0</td>
<td>3.89</td>
<td>0.54</td>
<td>0.12</td>
</tr>
<tr>
<td>49.3</td>
<td>4.00</td>
<td>0.61</td>
<td>0.17</td>
</tr>
<tr>
<td>54.0</td>
<td>3.80</td>
<td>0.66</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Sensitivity of Laurdan to lipid domains

A more promising probe for detecting phase domains coexistence in natural membranes is Laurdan (13-14). Laurdan excitation and emission spectra are sensitive to the phase state of the phospholipid bilayer (Figure 2). So the main advantage of this probe is represented by the possibility of using simple steady-state measurements.

Due to the peculiar sensitivity of Laurdan excitation spectra to the phase state of phospholipid vesicles, it is possible to photoselect different populations of Laurdan molecules by using excitation wavelengths at the edge of the spectrum (Figure 2A). This procedure can be regarded as the introduction of an initial "phase polarization" in the excitation of the sample. At 410 nm we preferentially excite Laurdan molecules in a surrounding composed of gel phase phospholipids, while at 340 nm we excite a similar amount of Laurdan molecules surrounded by both phases. Since also Laurdan emission spectra are sensitive to the phospholipid phase (Figure 2B), we can detect if the initial photoselection of the probe molecules has been maintained in the emission, i.e. if during Laurdan excited state an interconversion between the phases occurred.

Laurdan emission maximum shifts from 440 nm in the pure gel phase to about 490 nm in the liquid-crystalline phase. We have measured the fluorescence intensity ratio 440nm/490nm in phospholipid vesicles composed of both pure phases. Then the same measurements have been performed during the phospholipid transition or in vesicles composed of non-
miscible phospholipids, at a temperature where phase domains coexistence is expected. As a result, we observed that the initial photoexcitation of Laurdan molecules is not maintained in the emission, indicating a phase interconversion.

We have developed an analytical method to quantify the process, based on the definition of the "generalized polarization", GP:

$$GP = \frac{(I_b - I_e)}{(I_b + I_e)}$$

where $I_b$ represents the fluorescence intensity at 440 nm upon excitation at 410 nm, and $I_e$ represents the emission at 490 using the same excitation. The advantage of defining the "generalized polarization" for the analysis of the spectral properties of Laurdan is related to the well-known properties of the polarization which is a quantity that contains information on the dynamics of interconversion between different "states". In the classical definition, the "states" correspond to different orientations of the emitting dipoles with respect to the laboratory axes. In our case, higher GP values, closer to 1, correspond to the absence of interconversion between the phases.
Simulations using interconversion between the phases show that interconversion with a rate of 1/40 ns is needed to obtain the observed values of the GP.

To verify the occurrence of phase relaxation and to directly investigate the characteristic interconversion rate between the phases we performed time-resolved emission experiments (13). By multifrequency phase and modulation we measured Laurdan fluorescence at different emission bandpass (i.e. we measured the time evolution of the spectral shape) in mixed phase vesicles using an excitation wavelength of 325 nm. The results show...
the existence of two separate families of emitting molecules (Figure 3). The first is emitting at higher energies, with relatively blue spectra, while the second is emitting at lower energies, with red-shifted spectra. The second "red" family appeared 30-40 ns after the excitation, and contemporarily, the intensity of the "blue" family progressively decreased.

![Graph of Laurdan time resolved emission spectra](image)

*Fig. 3. Laurdan time resolved emission spectra in the equimolar mixture of dilauroyl- and dipalmitoyl-phosphatidylcholine at 20 °C. The phase and modulation data were acquired using a GREG 200 phase fluorometer (ISS Inc., Urbana IL) equipped with a He-Ne laser using the 325 line. Time resolved emission spectra have been obtained using the Global's Unithed software (University of Illinois, copyright 1988, Board of Trustees).*

We can then conclude that the detection of coexisting phase domains is possible, given the peculiar sensitivity of Laurdan excitation and emission spectra to the phase state of phospholipid bilayers. A method for the quantitation of the two phases has been also proposed by introducing the "generalized polarization". By measuring the time evolution of the emission shape and intensity of Laurdan fluorescence, the kinetics of interconversion between the phospholipid phases has also been determined.
ACKNOWLEDGEMENTS

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REFERENCES
