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Fluorescence Lifetime Distributions in Membrane Systems

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Membranes are complex biological systems that display heterogeneity at all spatial scales. At a molecular level, the heterogeneity arises from lipid and protein composition. At the cellular level, heterogeneity is due to membrane organization and large scale morphology. A quantitative evaluation of membrane heterogeneity at a microscopic level is very important for several fields of membrane studies. We have developed a method for the analysis of the decay of fluorescent membrane probes that can provide a quantity sensitive to membrane heterogeneity. This method is based on the analysis of the fluorescence decay using continuous lifetime distributions. The major challenge in the interpretation of the analysis results is in the identification, at a molecular level, of the mechanisms that influence the fluorescence decay. In this review we illustrate the principles of data analysis and we show examples of identification of the measured parameters with specific variables that affect membrane heterogeneity.

KEY WORDS: Membranes; fluorescence lifetime; DPH; lifetime distribution.

INTRODUCTION

The study of the structure and dynamics of membranes is an important field in biophysics. For many years, we have been interested in the determination of local structure and dynamics using fluorescence methods. In particular, our focus has been the study of water penetration into the membrane and the local order of the membrane as detected by fluorescent probes at different locations along the membrane normal. Using the fluorescent probe DPH (1,6-diphenyl-1,3,5-hexatriene), we have studied water penetration and membrane local structure in a variety of systems [1–6]. Although the DPH probe is generally used for determination of membrane order due to the preferential alignment of this probe along the lipid chains, several studies indicated that this probe also changes the fluorescence lifetime in different membrane environments [4,7–10]. Typical lifetime values in the gel phase of the bilayer are in the 10- to 11-ns range. In the gel phase, the lifetime increases as the temperature is increased, a peculiar behavior, since the lifetime of most fluorescence substances decreases as the temperature is increased due to the enhancement of nonradiative pathways. Instead, in the liquid crystalline state, typical lifetime values range from 9 to 6 ns. The reason for the lifetime change between the gel and the liquid crystalline state has been explained in terms of water penetration in the bilayer [4,11,12]. Experiments on small and large unilamellar vesicles (SUV and LUV) indicate that curvature and water penetration can also explain the experimental results [13]. The temperature dependence of the lifetime in the two phases has been explained in term of water penetration and the enhancement of the decay from the second electronic state caused by the difference in the dielectric constant [7,10,14]. The basic idea is that the fluorescence lifetime of DPH is strongly influenced by the dielectric constant of the surrounding medium [9,15], although alternative explanations have been proposed [16]. DPH prevalently resides in the membrane interior, presumably in the less-polar side, it has freedom to diffuse laterally in the membrane, but also to move rapidly along the

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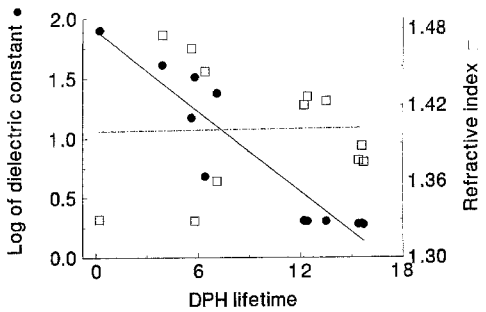

Fig. 1. Plot of the DPH fluorescence lifetime as a function of the log of the dielectric constant (left axis) and as a function of the refractive index (right axis). Data are from Refs. 4, 9, and 10.

membrane normal. In 1987, we developed an empirical method to analyze the fluorescence decay of DPH to account for the different locations of the probe in the membrane [1]. We reasoned that when DPH is deep into the membrane, the value of the fluorescence lifetime should be relatively long, similar to the value in nonpolar liquids. Instead, when DPH is closer to the membrane surface, the lifetime value should be shorter. If the movements along the membrane normal are slow with respect to the DPH decay rate, then the distribution of different molecular environments should be reflected in a continuous distribution of lifetime values. It has been reported by us and by several other groups that when the membrane dynamics is slow, such as in the gel phase of several phospholipids, the distribution of the lifetime values is relatively broad, and its average value (the center of the distribution) is shifted toward long lifetime values. Instead, in the liquid crystalline state, the lifetime distribution is relatively narrow and its average value is shifted to shorter lifetime values. To explain this effect, we have assumed that in the liquid crystalline state, the DPH probe senses the water penetration more and that fast movements with respect to the decay rate along the membrane normal make the average environment sensed by the probe more homogeneous. During the lifetime of the excited state, the probe will average many different environments. The environment with the shorter lifetime will dominate and this is the one mainly reported by the probe. The concept of fluorescence lifetime distribution is connected with two distinct phenomena: (i) The lifetime distribution arises from the sensitivity of the fluorescent probe to different environments, in particular to the different environments found in a membrane system. (ii) The dynamics of the probe, i.e., the rate of interconversion between different dielectric environments, must be slow or comparable to the excited-state lifetime. If the rate of interconversion is very fast, then a single average environment will be probed. Therefore, the analysis of the intensity decay can provide information on two important membrane parameters, namely the microheterogeneity of the probe environment and the dynamics of the microenvironment changes. Both those concepts have been exploited to study synthetic and natural membranes.

The physical origin of the differences in lifetime values when DPH molecules are located at different depths in the membrane is still under debate. Although we adhere to the proposal of Zannoni *et al.* [9] that the local value of the static dielectric constant is responsible for the lifetime differences, other explanations based on the local value of the index of refraction and on the relative orientation of the DPH molecule with respect to the membrane surface have been proposed (see the article by D. Toptygin and L. Brand in this issue). In Fig. 1 we report values of the DPH lifetime in different isotropic solvents obtained from our studies and from the literature. The value of the log of the static dielectric constant is represented on the left axis and the value of the refractive index is represented on the right axis. Inspection of this figure shows a correlation of the lifetime value with the static dielectric constant, but very little correlation with the refractive index of the isotropic solvent. This correlation does not exclude that in a particular series of homologous solvents, a better correlation can be found with the refractive index.

There are other situations that lead to a distribution of decay rates. One example studied in the context of protein folding is caused by energy transfer between donors and acceptors that can be at a distribution of distances [17,18]. In this case, there is a direct relationship between the distribution of distances and the distribution of decay rates. This situation can also arise in membranes in which the fluorescence intensity is, for example, quenched by the presence of other molecules. The random (or nonrandom) distribution of the quencher molecules can cause a distribution of decay rates. This concept does not apply to situations in which DPH is the only molecule in the membrane, or when specific acceptors for the DPH fluorescence are absent, which are the conditions for most of the experiments we are discussing in this article.

Of course, DPH is not the only molecule that displays a distribution of lifetimes in membranes. Other fluorescent dyes have been studied as well [19,20]. For example, TMA-DPH shows broader lifetime distributions than DPH, possibly because it is located in a region of large changes of the dielectric constant. Interesting probes are the isomers of parinaric acids, which, as commonly observed in conjugated polyenes, also display a

complex multimodal lifetime distribution [11]. In practice, every fluorescent probe, when studied with sufficient resolution, displays a relatively large lifetime heterogeneity in membrane systems.

FLUORESCENCE LIFETIME DISTRIBUTIONS

The fluorescence decay from a single homogeneous population in the absence of excited-state reaction is described by a single exponential decay term with characteristic time τ,

$$I(t) = \alpha\, e^{-t/\tau} \tag{1}$$

The decay from a heterogeneous population composed of n different noninterconverting molecular species can be described by a sum of exponential components,

$$I(t) = \sum_{i=1}^{n} \alpha_i\, e^{-t/\tau_i} \tag{2}$$

The decay time τ_i of each component depends on the photophysical characteristic of the molecular species. Each species contributes to the intensity decay by a factor α_i. The average fluorescence intensity is given by the integral over time of the intensity decay,

$$\langle I \rangle = \int_0^\infty \sum_{i=1}^{n} \alpha_i\, e^{-t/\tau_i}\, dt \tag{3}$$

The fractional contribution of the component i to the steady-state fluorescence intensity is given by the following expression:

$$f_i = \frac{\alpha_i \tau_i}{\sum_{j=1}^{n} \alpha_j \tau_j} \tag{4}$$

In the continuous lifetime distribution representation of the decay, the sum in Eq. (2) is substituted by an integral

$$I(t) = \int_0^\infty \alpha(\tau)\, e^{-t/\tau}\, d\tau \tag{5}$$

The function $\alpha(\tau)$ represents the distribution of lifetime values. It is possible to describe the decay using the fractional fluorescence contribution to the steady-state intensity rather than the preexponential factor, using definitions similar to that used in expression (4).

One of the most challenging problems in data analysis is the determination of the exact function $\alpha(\tau)$. Generally, a simple inversion process cannot be applied to relationship (5) due to the ill-defined nature of the problem. At least two different approaches have been proposed. In one case, the basic idea is to apply some mathematical procedure to extract the function $\alpha(\tau)$; in the other case, it is assumed that the particular functional form of the distribution is known and then the parameters describing the function are recovered using standard fitting procedures. This last approach was initially used, for example, for the recovery of a Gaussian distribution of distances in energy transfer experiments. Instead a different analysis method assumes no *a priori* knowledge of the functional form of $\alpha(\tau)$. Among these methods, the most successful are the maximum entropy method [21,22] and the exponential series approach. In the following, we will discuss analysis methods in which a form is assumed for the lifetime distribution and the parameters of the functional form are associated with some physical property of the membrane [23,24]. The most commonly used functional forms for the lifetime distribution (in terms of fractional intensities) are the Lorentzian and the Gaussian forms:

$$f(\tau) = A/1 + (\tau - \tau_0)^2/w^2 \tag{6}$$

$$f(\tau) = A\, e^{-(\tau-\tau_0)^2/2w^2} \tag{7}$$

were τ_0 is the center of the distribution and w the standard deviation (Gaussian) or the full-width at half-maximum (Lorentzian) of the distribution. Frequently, the decay is described by a sum of two or more distributions, i.e., multimodal distributions. In this case, it is customary to represent the total decay by a sum of terms such as those of Eq. (5), each multiplied by a factor proportional to the fractional contribution to the total fluorescence intensity of that term to the total distribution. This representation is useful when two or more independent molecular species contribute to the total decay. Then a plot of the multimodal lifetime distribution directly shows the different components with an amplitude proportional to their contribution. Note that it is the amplitude and not the area under the distribution that represents the fractional intensity of each molecular species. This representation has led to frequent discussions, since it is argued that the area under the curve should better represent the contribution to the fluorescence. Although this can be a correct way to plot the lifetime distribution, in many situations there are lifetime components that can arise from impurities or that are better represented by a single-exponential decay. In the area representation, a single-exponential decay will have infinite (or at least very large) amplitude and will render the representation of the multimodal decay unfeasible if exponentials and distributions coexist. A different problem arises when a representation in terms of preexpo-

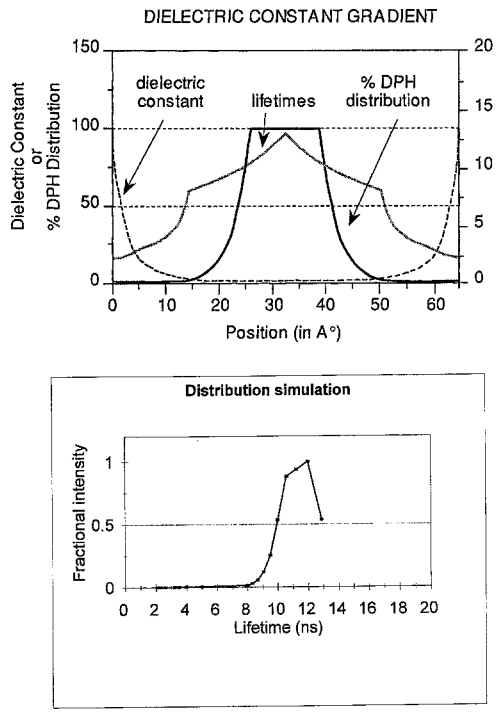

Fig. 2. (A) Schematic representation of the dielectric constant, lifetime value, and relative concentration of DPH molecules as a function of the distance from the bilayer surface. (B) Lifetime distribution calculated from the plots of part A.

nential factors rather than fractional fluorescence intensities is desired. In this case a transformation from molecular species to the contribution to the total intensity is necessary using expressions such as Eq. (4).

THE MEANING OF THE LIFETIME DISTRIBUTION

The analysis of the intensity decay using a continuous distribution of lifetimes is now quite common in the literature on membrane systems [1,2,21,24–37]. What really matters is not so much the method for data analysis or the representation of the decay, but rather the meaning that is attributed to the parameters describing the lifetime distribution. For the purpose of presentation, we will consider the decay represented by a Lorentzian distribution that is described by two parameters: the center and the width of the distribution. It is common practice to associate the center of the lifetime distribution with the average lifetime value. This is not strictly true, because the Lorentzian distribution extends to infinity, but negative lifetime values are generally not allowed. Also, the weighting of the tails of the distribution is only polynomial for the Lorentzian distribution and the distribution must be truncated to some arbitrary value to make physical sense. However, in most cases the distribution is narrow enough that the above considerations have only minor importance. The width of the lifetime distribution is generally interpreted as indicative of the heterogeneity of the sample. As we mention in the introduction, the concept of the distribution width, as well as the distribution center, should not only relate to the heterogeneity of the system, but also to the dynamics of interchange between different environments. For example, a narrow lifetime distribution width can arise from a very homogeneous system or from a heterogeneous system with very fast dynamics. To distinguish between these two possibilities, it is possible either to measure directly the dynamics of the system by measuring, for example, the decay of the emission anisotropy or to change some parameters such as the temperature that can affect the dynamics of the system. If the dynamics of the system is slow, then the distribution width can be empirically correlated to the heterogeneity of the system. This correlation has been carried out in several systems [2,33–36]. In the gel phase of the bilayer structure, it is generally found that the lipid dynamics is slow on the fluorescence time scale of most fluorescent probes. The general finding is that the lifetime distribution is broad. This indicates a relatively large heterogeneity of the fluorescent probe environment. To better define the heterogeneity, we need to formulate a specific model. For DPH, it has been proposed that fluorescence lifetime is a direct function of the dielectric constant. In principle, it should be possible to associate each lifetime component with a particular value of the dielectric constant. If we make the hypothesis that the differences in dielectric constant depend on the water penetration in the membrane, and if we model how the dielectric constant varies as a function of the distance from the membrane surface, then we can map the lifetime distribution into a distribution of locations of the DPH molecules along the membrane normal. Figure 2 shows in a diagrammatic form this concept. In Fig. 2A, a dielectric constant profile is assumed. At the membrane surface the dielectric constant is 80 and in the membrane interior is about 1.5. The model assumes that most of the dielectric constant drops in the first 5 Å from the membrane surface. An inverse relationship is assumed between lifetime and dielectric constant: In water, the lifetime is set to about 2.5 ns and in the membrane interior, where the dielectric constant is 1.5, the lifetime is fixed at 13 ns. The dashed line in the figure is the map of lifetime values in the membrane due to the gradient of dielectric constant. To calculate the lifetime distribution, we must assume a dis-

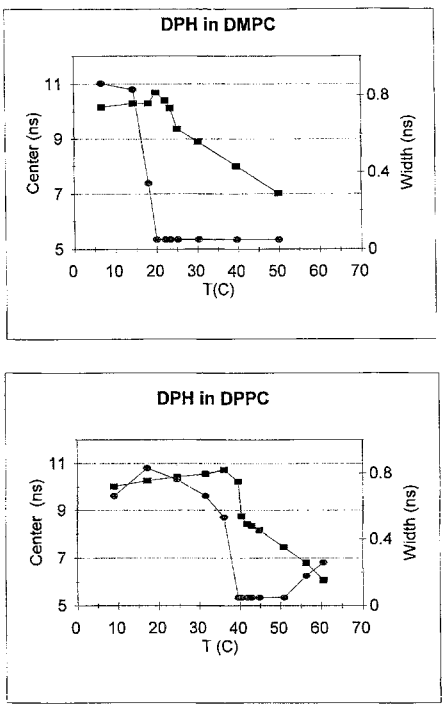

Fig. 3. (A) Center (■) and width (●) of the Lorentzian lifetime distribution for DPH in DMPC. The decay has been resolved using two Lorentzian distributions. The main component, about 95% of the fluorescence intensity, is represented in the figure. The second, small component has an average lifetime of about 2.5 ns and a width of about 1 ns. (B) Center (■) and width (●) of the Lorentzian lifetime distribution for DPH in DPPC. The decay has been resolved using two Lorentzian distributions. The main component, about 95% of the fluorescence intensity, is represented in the figure. The second, small component has an average lifetime of about 2.5 ns and a width of about 1 ns.

tribution of DPH molecules in the membrane. The solid curve labeled % DPH distribution represents the relative concentration of DPH in the membrane. Figure 2B shows the lifetime distribution which results from the assumption of Fig. 2A. For this simulation, the DPH molecules are considered fixed in position during the excited-state lifetime. This condition should simulate the gel phase of the membrane.

In the liquid crystalline state, which is the state commonly found in biological membranes, the correlation between lifetime distribution and the heterogeneity of the probe environment cannot be obtained in such a simple way. In the liquid crystalline state, the membrane dynamics is sufficiently fast to affect the parameters of the lifetime distribution. Therefore, either the distribution is very narrow or the distribution reflects other environmental factors such as heterogeneity in the membrane plane [35]. One common example is the existence of regions or domains in the membrane of different organization or different concentration of some host molecule, of sufficient stability not to change during the excited-state lifetime [33,34]. It is clear from this discussion that the value of the probe lifetime can be chosen to detect different kinds of dynamic phenomena in membranes.

In summary, in the current literature on membrane systems, the lifetime distribution is either used to represent a well-defined physical model, or is used as an empirical indicator of local microheterogeneity and dynamics of the membrane system.

LIFETIME DISTRIBUTION STUDIES

In this section, we discuss a few relevant examples of the application of the fluorescence lifetime distribution of DPH to different membrane systems. The literature on applications of the lifetime distribution analysis applied to the fluorescence decay of different membrane probes is relatively vast. In this review, the examples chosen are intended to explain different aspects of the lifetime distribution analysis rather than a complete list of the literature on the subject.

The first study of lifetime distributions in membrane systems was that of Fiorini *et al.* [1] using the DPH probe. Two model membrane systems were studied, DMPC and DPPC multilamellar liposomes. The report includes studies as a function of temperature from 10°C to about 60°C. For both membrane systems, the width of the lifetime distribution was relatively broad (about 1 ns) below the phase transition. During and above the phase transition, the distribution became very narrow. These results were interpreted in terms of a gradient of dielectric constant in the gel phase. This gradient is stable during the excited-state lifetime. Above the lipid phase transition, the mobility of the DPH molecule increases to the point that only an average environment can be detected during the excited-state lifetime. Due to this motional effect, the lifetime distribution collapses. Figure 3 reports the lifetime distribution center and width for DPH in DMPC and DPPC. The width of the lifetime distribution collapses a few degrees before the phase transition. The original interpretation of the 1987 article is still valid as more evidence is now available about the extent of water penetration in the membrane and on the mobility of the DPH molecule.

In another series of studies, the same approach was applied to the study of the erythrocyte membrane. It was found that at 40°C the lifetime distribution is relatively

narrow. However, when the membranes were partially depleted of cholesterol, the lifetime distribution width increased, pointing out the effect of cholesterol in homogenizing the membrane and possibly depleting the membrane of water. In the same article, the effect of cholesterol was observed in egg phosphatidylcholine liposomes at 40°C. At this temperature, the lifetime distribution was relatively broad, but it markedly decreased upon addition of 5 mol % of cholesterol. The same addition of cholesterol did not produce a change of the distribution center. This example shows how the changes of the distribution width can be interpreted as due to changes of membrane heterogeneity and dynamics induced by cholesterol.

Recently, a new method that exploits the concept of DPH fluorescence lifetime distribution has been developed for the detection of oxidative damage induced in the lipid components of biological membranes by low doses of ionizing radiation [5,6,38]. The fluorescence decay of DPH was modeled using a Lorentzian distribution. The width of the distribution was found to correlate with the oxidative damage produced in cells. In particular, an inverse linear relationship was found between the width of the lifetime distribution and the log of the radiation dose. The idea is that the slope of the dose–radiation curve is related to the number and position of the unsaturated acyl residues. Following exposure to ionizing radiation, hydroperoxides are formed at the core of the membrane [39]. It is proposed that the disorder introduced by these new residues induces water penetration, hence quenching of the DPH fluorescence. As a result, only fluorescence from the deeper part of the membrane can be observed. Also the defects along the acyl chain should prevent lateral movement that further induces the collapse of the lifetime distribution. In this example, the effect on the lifetime distribution width due to water penetration and immobilization of the DPH molecule in the membrane interior are used as an indicator of radiation damage to cells.

The group of A. Hermetter in Graz has used extensively the concept of lifetime distributions of different DPH derivatives to study lipid organization and lipid protein interactions [27,33,34,40]. For example, the fluorescence lifetime distribution of phosphatidylcholine and sphingomyelin DPH derivatives were employed to discriminate between the interaction of lipids with low-density lipoprotein (LDL) and lipoprotein (a) [Lp(a)]. It was found that the lifetime distribution center of both DPH derivative probes was very similar except for DPH-PC in Lp(a), which was shifted to a longer lifetime value, indicating a less polar environment of the PC in Lp(a) when compared to LDL. The distribution width of DPH-PC in Lp(a) was also broader than in LDL. Using the width of the lifetime distribution as an indication of the heterogeneity of the probe environment, it was concluded that LDL provides a more homogeneous environment that Lp(a). On the other hand, no difference was found for the sphingomyelin in the presence of the two types of proteins.

The group of C. Stubbs in Pennsylvania has also used the concept of lifetime distribution in a very similar way [35,36]. The width of the distribution is used to detect interactions between lipids and proteins. The basic idea is that two different types of information can be obtained from the lifetime distribution studies: namely, the average probe polarity and the heterogeneity of the probe surroundings. In this kind of study, there is no attempt to correlate the lifetime distribution parameters to a specific physical model, but rather the existence of the distribution per se is used as evidence of a multiplicity of environments for the probe.

In conclusion, membranes are complex systems that display a large microheterogeneity with different length and time scales. The distribution analysis approach of the fluorescence decay of membrane probes can provide new information on the microheterogeneity and dynamics of complex membrane systems.

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